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It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR

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THE APPARENT OXIDATION-REDUCTION POTENTIALS OF BRIGHT PLATINUM ELECTRODES IN SYNTHETIC MEDIA CULTURES OF BACTERIA

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The study of oxidation-reduction potential changes in culture of various organisms has been pursued by many investigators since Potter (1911) first noted that the electrode potential of an inoculated medium was more negative than a sterile control. The extensive literature which has ensued contains results of determinations obtained almost entirely by the use of complex media of unknown composition. A few workers (Elema, *et al.*, 1934, Allyn and Baldwin, 1932, Thorne and Walker, 1936) have used media of known chemical composition as a substrate for the growth of soil and related organisms in redox studies. Thus far, however, to the writer's knowledge no attempt has been made to make similar studies with pathogenic or potentially pathogenic bacteria. As a result of the recent work on growth accessory substances or vitamin requirements (Koser, *et al.*, 1935, 1936; Sahyun, *et al.*, 1936; Knight, 1937 and Mueller, 1937a, b) it is possible to make potential measurements of growing synthetic-medium cultures of these latter forms.

The potentiometric method has been found superior to the colorimetric method for measuring potentials of this type. Preliminary determinations made with a conventional type K potentiometer bridge have shown such an arrangement to be entirely unsatisfactory for measurements in nutrient broth and synthetic media. The range of values obtained by consecutive readings was too wide to allow significance to be attached to them. This undoubtedly was due, in part, to polarization phenomena. A vacuum tube null-point instrument was con-

structed which eliminated polarization effects and gave identical consecutive readings in poorly poised systems. Despite this, however, discrepancies between duplicate electrodes and identical culture vessels were still encountered in both nutrient broth and synthetic media cultures. These irregularities appeared to be of sufficient magnitude and importance to warrant further study. From theoretical considerations there were several possible factors that might be involved: (1) Since a certain amount of stratification is known to occur in the growth of organisms in liquid media it seemed of value to investigate the effect of agitation of the medium. (2) In view of somewhat conflicting statements in the literature regarding electrodes, several types were used. (3) The poorly poised condition of simple culture media solutions suggested that appropriate poising substances might be tested. (4) The control of oxygen tension is obviously of importance both because of interference with hydrogen transfer and because of an oxygen electrode effect. In the present paper are reported comparative data obtained in a study of the relation of these factors to the apparent oxidation-reduction potentials produced in broth and synthetic media. Although it is recognized that blank platinum electrodes may in the presence of hydrogen give a hydrogen electrode effect, this aspect of the problem was not investigated directly since the test organism, *Escherichia coli*, ordinarily liberates a certain amount of this gas in its growth processes.

METHODS

The vacuum tube circuit[1] is shown in figure 1. The vacuum tube heater voltage is set to approximately four volts by means of the 20-ohm variable resistance. Adjustment is made in the plate current line by manipulation of the potentiometers inserted therein so that no deflection of the galvanometer occurs when the tap key *A* is closed. Approximately 0.1 milliampere is read on *M*. During this period of primary balance the control grid is isolated and floating and as a result acquires a potential value

[1] This circuit is a slight modification of one suggested by Dr. Otto H. A. Schmitt of Washington University, St. Louis.

at which the grid current is practically eliminated. The maximum grid current at any time was 10^{-11} ampere. Finally this grid is biased to this latter value by closing the special air-insulated switch B, closing C and adjusting the 6000-ohm and 600-ohm potentiometer resistances. The point of balance is again detected by using the key A. Switch B is now opened and the unknown half cell is connected to one pole of X and the saturated calomel fiducial cell to the other pole. The former cell is connected to the latter by means of an agar potassium-chloride bridge using a saturated potassium-chloride liquid junction. The

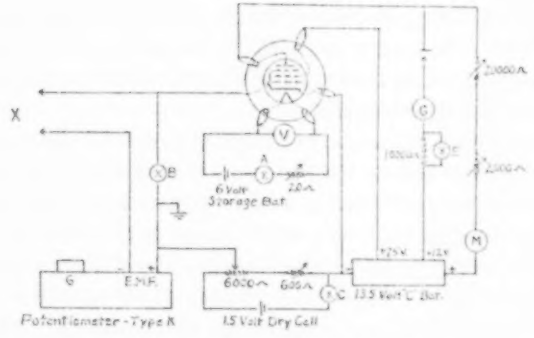

FIG. 1. VACUUM TUBE CIRCUIT

M, 0–1 milliammeter; *V*, 0–6 voltmeter; *G*, galvanometer; *X*, unknown half cell and reference half cell; *B*, air insulated switch; *A*, *C*, and *E* are simple toggle switches. Type 38 R. C. A. tube.

potential of the complete cell is then obtained as a direct reading on the potentiometer when *G* again shows no deflection. The calomel cell was prepared from electrolytic calomel, recrystallized potassium chloride and redistilled mercury. On checking against various buffers it was found to have the value 0.2364 volt at 37.5°C. A Leeds and Northrup lamp and scale type galvanometer having a current sensitivity of 0.025 microampere was used in most of the experiments. The performance of this instrument was checked against the more sensitive reflecting type having a sensitivity of 0.0005 microampere. The results were identical in all cases.

The electrodes were connected with the measuring apparatus through small mercury-containing steel cups embedded in high-melting-point paraffin. This material offered a suitable means of insulation because of its high resistance. Long, rectangular blocks of paraffin containing these cups were supported by the upper edges of the constant temperature bath in which the culture cells were incubated at 37.5 ±0.02°C. The culture vessels were 180 ml. electrolytic beakers without pours. These were closed with alkali-treated rubber stoppers containing five holes, two for electrodes, one for the agar potassium chloride bridge, one to allow for filling with medium, and a central one for the stirring arrangement. The latter was sealed either with mercury or oil. Mercury appeared to be more efficient and was easier to clean out than the oil; so it was used in the majority of the agitation experiments.

Electrodes were prepared by sealing into soft glass tubing of the desired size. Before the seal was made, a piece of 28-gauge platinum wire was silver-soldered to the part of the electrode entering the tube so that this small gauge wire could be brought out the other end and the glass sealed about it. This gave a glass tube sealed at both ends carrying the contact wire to which, on the outer end, a small piece of copper wire was silver-soldered for making contact with a mercury cup. An electrode of this type was easy to clean and handle.

For cleaning the electrodes, hot chromic acid, nitric acid and *aqua regia* were tested. As far as could be detected no one method was superior. The nitric acid method has been used in practice because the action of *aqua regia* is somewhat vigorous on fine gauze or foil electrodes. Those cleaned by heating gave no better results than were secured by the other methods.

The stirring mechanism, of the mechanical type, was operated by a ratio motor with output of 86 r.p.m. From this, by a series of pulleys and belts, the stirrers were operated in from one to ten cells simultaneously at a speed of approximately 130 r.p.m.

All synthetic media were prepared by weighing the requisite C.P. or Reagent chemicals on an analytical balance and adding to sterile re-distilled water. The water-clear solution was then filtered through a Seitz pad or Berkefeld N candle and incubated

to test for sterility. The basic synthetic medium, a slight modification of that of Koser and Saunders (1935), was composed of Na_2HPO_4 1.4 grams, KH_2PO_4 1.0 gram, NaCl 2.0 grams, $MgSO_4$ (anhy.) 0.1 gram, l-asparagine 3.0 grams, l-tryptophane 0.2 gram, d-glucose 2.0 grams and re-distilled water 1 liter. The final pH was 6.9. Standard sodium hydroxide was used to adjust the pH when additions such as cysteine hydrochloride were made. The nutrient broth used was composed of beef extract (Swift) 3.0 grams, peptone (Difco) 10.0 grams, NaCl 5.0 grams and re-distilled water 500 ml. This was adjusted to a pH of 6.9, sterilized in the autoclave, and to it added 500 ml. of sterile phosphate buffer (pH 6.9 at 37.5°C.). The buffer was present in a final concentration of M/30.

The culture vessels containing only the electrodes were sterilized by autoclaving. Before the electrodes dried, the medium, in 100 ml. quantities, was pipetted into the vessels. The agar potassium-chloride bridges were filled aseptically, inserted into the bung, and held in place by a section of close-fitting rubber tubing.

In the anaerobic experiments, commercial water-pumped cylinder nitrogen was purified by bubbling through sodium hydroxide solution and concentrated sulfuric acid, then run through a heavy wall pyrex combustion tube held in an electric furnace. The combustion tube was packed with small-gauge copper oxide wire and the latter was reduced in a stream of hydrogen before each experiment. Precautions were taken to blow all hydrogen out of the combustion tube before the culture vessels were connected. From the combustion tube the purified gas was led through a trap and then into a Y tube extending across and above the two rows of culture vessels. The nitrogen was bubbled through the medium in each vessel at approximately an equal rate. To accomplish this a piece of small-bore glass tubing was run through the center of each rubber bung and connected, using heavy-wall rubber tubing, to another piece of glass tubing of the same bore which projected at right angles from the nitrogen line. The two ends of glass tubing were approximated so that the chance of oxygen diffusing through the connection was lessened. Both pieces of tubing were stoppered

with a small piece of non-absorbent cotton. It is realized that these conditions do not yield absolute anaerobiosis, but it was felt that they were sufficient for the desired studies.

In every experiment all vessels were checked microscopically and culturally for contamination at the end of each determination. Counts were also made at the conclusion of an experiment to determine the total number of bacteria per ml. The vessels were labeled alphabetically in order to prevent confusion when taking measurements.

A carefully checked stock strain of *Escherichia coli* which did not ferment sucrose was used in all determinations. Inoculations were made directly into the medium from a twenty-four-hour agar slant culture. One hundred ml. aliquots were then pipetted out and, thus, each inoculated culture vessel had practically an equal number of organisms. However, no further attempt was made to standardize the number of inoculated organisms for it was thought that if good agreement was obtained consistently in individual experiments, the duplication of experimental results should not be difficult.

Every electrode was examined for flaws at the conclusion of each experiment and all were checked against one another by measurement of dilute solutions of ferrous-ferric salt mixtures. If defects were noted the results for the corresponding vessel were discarded from the data.

All experiments were performed at least three times. In each determination an average of ten separate culture vessels were used, each of which contained duplicate electrodes.

The hydrogen ion concentrations of the various media were determined before and at the end of the incubation period by means of a glass electrode.

EXPERIMENTAL RESULTS

Stationary synthetic medium cultures

Under conditions wherein no agitation of the substrate occurred, the experimental results were at variance between vessels containing identical electrodes, medium, and number of inoculated organisms. In figure 2 typical time-potential curves ob-

tained by using platinum wire spiral electrodes are shown. The double-line curve appearing under each letter represents a plotting of values for individual identical duplicate electrodes in the same culture vessel. Representatives were chosen from neither extreme, being selected from a group of ten vessels all of which were incubated at the same time. Other identical experiments always gave similar variations. The maximum difference shown,

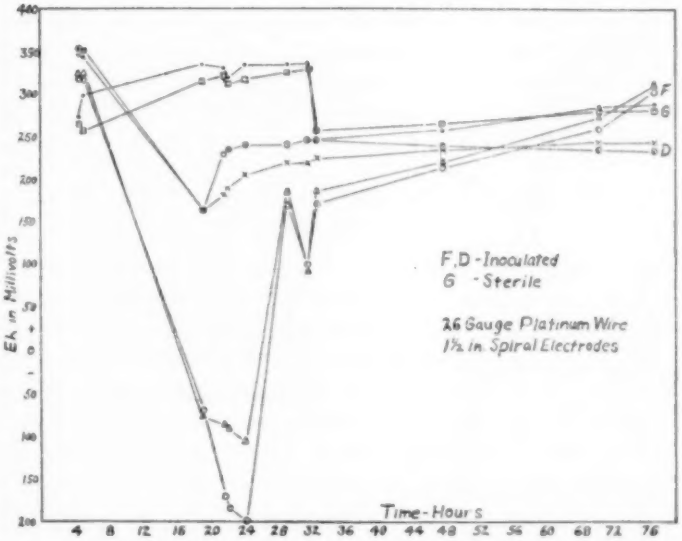

FIG. 2. TIME-POTENTIAL CURVES OBTAINED IN THE BASIC SYNTHETIC MEDIUM UNDER STATIONARY CONDITIONS
Each curve represents values for a single electrode

which is approximately 444 millivolts, exists between values obtained in separate vessels while the maximum between duplicate electrodes in any one vessel is 95 millivolts. In the majority of cases, readings became more nearly concurrent after the culture had been incubated thirty-six hours. There was no indication, when growth comparisons were made, that these or subsequent irregularities were due to differential growth in various vessels.

Results obtained by using other forms of platinum electrodes

showed a shift in the general oxidation-reduction intensity which will be discussed later, but discrepancies were still apparent.

Intermittently agitated synthetic medium cultures

Since the irregularities shown in the previous experiment are not readily explicable on the basis of mechanical defects or

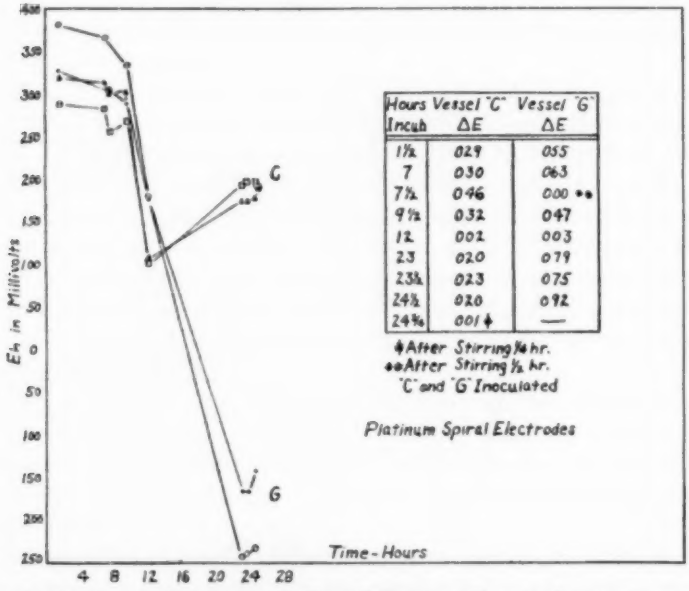

FIG. 3. EFFECT OF AGITATION ON TIME-POTENTIAL VALUES OBTAINED IN THE BASIC SYNTHETIC MEDIUM

ΔE, in millivolts, is equal to the difference between duplicate electrodes in the same vessel. Each curve represents values for a single electrode.

methods used in cleaning the electrodes, the possibility is to be considered that localized growth, collection of gas bubbles, or temporary poisoning of one electrode might account for some questionable potential readings.

The plotted values in figure 3 are typical for cultures stirred for several short intervals during incubation. ΔE, in millivolts, is equal to the difference between duplicate electrodes in the

same culture vessel at any one time. After seven hours' incubation under stationary conditions the electrodes, of the platinum wire spiral type, in vessel *G* gave a ΔE value of 63 millivolts. As a result of agitating for one-half hour ΔE was equal to zero. Likewise in vessel *C* the ΔE value was changed from twenty millivolts at twenty-four and one-half hours to one millivolt after stirring for fifteen minutes.

Better agreement, not necessarily absolute, was obtained between like duplicate electrodes in the same vessel under conditions of agitation, although there still remained a considerable difference among individual vessels containing the same medium. This again could not be accounted for by differences in growth or electrode defects.

Platinum electrode types in synthetic medium cultures under conditions of agitation

During the course of these experiments, various types of electrodes—platinum wire pin-point and spiral forms—as well as platinum foil and gauze types were tested. The time-potential relationships obtained in the basic synthetic medium plus 0.10 gram of cysteine hydrochloride are presented in figure 4. The curves in this figure were plotted from averages derived from duplicate electrodes in each vessel. In many cases ΔE values were rather large, so the mean is of questionable significance. These ΔE values ranged from 100 millivolts to zero millivolt, with approximately the same degree of irregularity appearing in both sterile and inoculated vessels.

The high values given by foil electrodes in the sterile medium, as shown by curves *E* and *D*, are typical for these and for gauze types. The potential in the same sterile medium was approximately 200 millivolts higher when determined by means of foil electrodes in contrast with readings with wire electrodes. The regularity of *E* and *D* as shown in the graph was not always as pronounced. In the inoculated medium, the wire electrodes and foil electrodes did not give the marked differences that were obtained in the sterile medium.

There was no agitation of the cultures between the twelfth

hour and the twenty-ninth hour. This results in the appearance of a trough in the curve at this time. In other experiments where cultures were not agitated or were constantly agitated throughout the entire period, the greatest reducing intensity appeared earlier. The inoculated vessels, again, regardless of

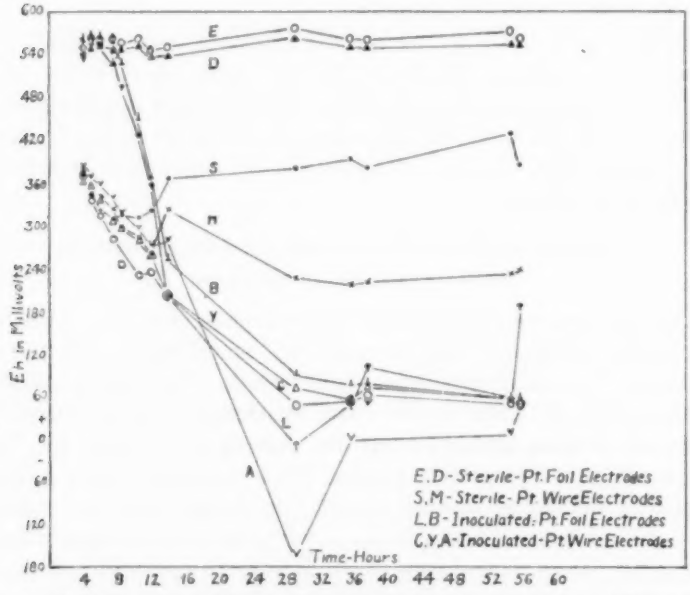

FIG. 4. TIME-POTENTIAL RELATIONSHIPS OBTAINED IN SYNTHETIC MEDIUM BY PLATINUM FOIL AND WIRE SPIRAL ELECTRODE TYPES UNDER CONDITIONS OF AGITATION

Each curve represents an average of the readings obtained from duplicate electrodes in the same vessel.

the electrode type, showed an approaching similarity of values after thirty-six hours' incubation.

Since the presence of cysteine in the medium might be considered to be a cause of the irregular values, the experiment was repeated using the basic synthetic medium without cysteine. Similar results were obtained. In the experiment shown in figure 4 and in additional experiments of a similar nature, there

was no case in which any one electrode type appeared to give more consistent results than the other types.

Nutrient broth cultures compared with synthetic medium cultures under conditions of agitation

Previous workers, using broth and more complex media of unknown composition, have reported close agreement between duplicate electrode and duplicate vessel readings. The nutrient broth medium was used in these experiments in order to compare the results obtained in it with those in a synthetic medium.

The data obtained by using broth medium were consistently in better agreement. The majority of the ΔE values were in a range of 0 to 5 millivolts, with an occasional higher value of 20 millivolts. The difference between culture vessels was of the order of 5 to 10 millivolts. At times, apparently when metabolic changes were quite rapid, this difference was in a few instances somewhat greater. The results here, in contrast to those obtained in experiments using synthetic media, were fairly consistent throughout the entire period of incubation.

Semi-poised synthetic medium cultures

In the data thus far presented there is a suggestion that the irregularities still prevalent in a synthetic medium culture are due either to polarization of electrodes or to the absence of sufficient oxidizing-reducing material to give stable true values in the presence of oxygen. Polarization of the electrodes due to the measuring apparatus was ruled out because the irregular values were reproducible over a period of time and in different experiments. The electrodes in question, when checked against an inorganic oxidation-reduction system, always gave extremely regular results independent of type, shape or size of the metal surface. These discrepancies appeared, then, to be due to a culture medium poorly poised in the presence of air. Consequently, a reversible inorganic oxidation-reduction system was added to the synthetic medium in concentrations small enough so as not to alter the growth of the organisms perceptibly. Preliminary results, obtained by growing the test bacterium in the

synthetic medium and adding to the vessels (after irregularities were noted), small concentrations of potassium ferricyanide, were encouraging.

In figure 5 are recorded results obtained by allowing the organism to grow in the synthetic medium containing a 0.00031 *M* concentration of $K_3Fe(CN)_6$. Growth was excellent, and the

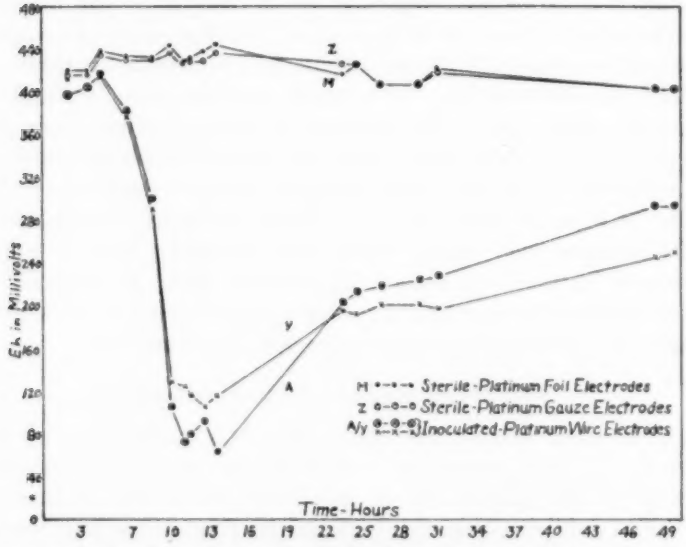
Eh in Millivolts
Time - Hours
H Sterile - Platinum Foil Electrodes
Z Sterile - Platinum Gauze Electrodes
A, y Inoculated - Platinum Wire Electrodes

FIG. 5. TIME-POTENTIAL RELATIONSHIPS OBTAINED IN THE BASIC SYNTHETIC MEDIUM PLUS 0.00031 *M* CONCENTRATION OF $K_3Fe(CN)_6$ UNDER CONSTANT AGITATION

Each curve represents an average of the readings obtained from duplicate electrodes in the same vessel.

results were regular for the first ten hours of incubation. Such variations as did occur were of a small magnitude. In the sterile medium ΔE values were of the order of 2 millivolts, while in the inoculated medium the maximum variation was greater (20 millivolts). Experiments in which the concentration of $K_3Fe(CN)_6$ was increased gave almost perfect data. However, growth in such cases was retarded to a slight extent. It should

be noted that this poising of the medium dispelled differences in electrode types.

Synthetic medium anaerobic cultures

In view of certain peculiarities in the electrode effects previously noted, it seemed likely that some irregularities could be

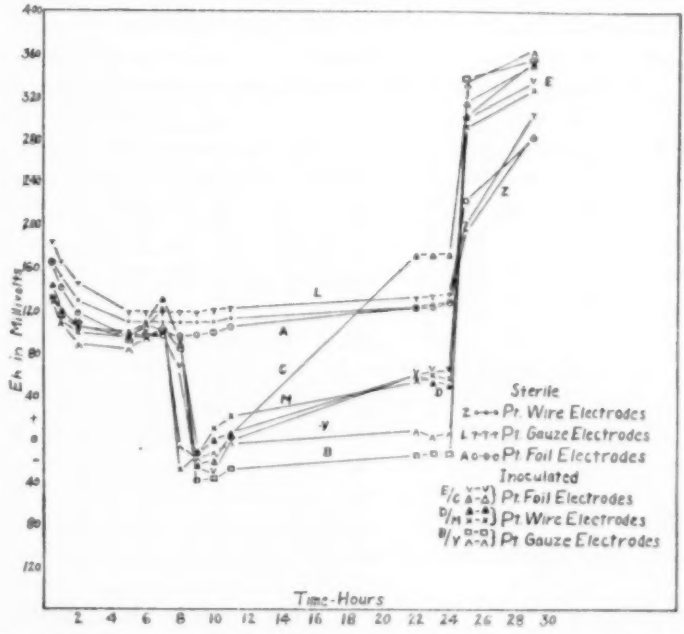

FIG. 6. TIME-POTENTIAL CURVES OBTAINED FROM THE BASIC SYNTHETIC MEDIUM UNDER ANAEROBIC CONDITIONS

Each curve represents an average of the readings obtained from duplicate electrodes in the same vessel. Pure oxygen was bubbled through the cultures beginning with the twenty-four hour period.

eliminated by removing atmospheric oxygen from the culture medium. The basic synthetic medium was chilled before being put into the culture vessels so that most of the oxygen could be removed by the stream of nitrogen before the temperature rose to a point where growth would become vigorous. One hour was

allowed for temperature equilibration. This was ample as judged by a control vessel. Time was counted from the end of this period.

Results obtained are plotted in figure 6 and ΔE values are given in table 1. It is to be noted, in contrast to aerobic determinations, that electrode values were regular for the first seven hours. After this time a rather sudden drop toward negative

TABLE 1

ΔE values in millivolts for basic synthetic medium anaerobic cultures

HOURS	STERILE			INOCULATED					
	Z	A	L	M	D	C	E	Y	B
½	1	3	10	1	2	3	2	2	2
1	2	2	6	3	5	3	2	0	2
2	2	2	4	2	2	2	1	0	1
5	3	2	1	0	2	0	0	0	1
6	2	2	1	0	1	0	1	0	1
7	2	2	1	1	2	0	0	0	0
8	2	1	1	4	7	1	0	12	7
9	2	1	0	6	11	1	4	7	19
10	1	2	1	10	29	1	7	3	39
11	2	0	0	11	34	2	10	2	56
22	1	0	0	10	40	1	9	29	71
23	2	0	0	2	40	1	10	25	70
24	1	0	0	5	43	2	9	28	78
25	19	0	3	0	11	8	0	26	22
29	11	7	17	3	35	1	0	25	21

potentials occurred. This latter phenomenon was practically constant as to time and rapidity of appearance.

The sharp rise to positive values occuring at twenty-four hours (fig. 6) was due to pure oxygen being bubbled through the cultures at this time. This introduced irregularities, especially in the sterile medium, as is shown by table 1.

DISCUSSION

The majority of workers who have studied redox potential changes in bacterial cultures have used broth or more complex

media. Using synthetic media it has been difficult to obtain such reliable data as could be obtained in a broth medium.

The use of synthetic media offers numerous possibilities for clarifying growth-potential relationships; but at the same time, many difficulties present themselves. The data show many of these irregularities and methods by which they may be circumvented.

In a non-agitated synthetic medium culture it is shown that anything resembling concurring values can only be obtained following thirty-six or more hours of incubation. After this time very little growth, in comparison to the earlier hours, occurs, so the measurements are not of what actually happened during the period of active proliferation but are of the end result of the entire process. As to whether the first portion of the time potential curve is of importance there is some disagreement. Under aerobic conditions of growth the point where the curve begins to become parallel with the abscissa axis would be dependent upon the number of organisms in the inoculum, upon the amount of poising agent present in the medium (in most cases this has been an unknown and disregarded factor), upon the oxygen tension of the medium and upon the reducing capacity of the bacterial redox systems. Any negligence in the consideration of these first three factors would make all points on the curve quantitatively erroneous. Where anaerobic conditions have been maintained, the effect of an important factor, oxygen, has been removed.

Constant mechanical agitation, under aerobic conditions of growth, is shown to be of some importance in obtaining better agreement of results. This must be the consequence of better equilibration due to the removal of poisoning material and zone effects. The observations suggested that there should be a tendency for electrodes to become sluggish due to coating with cells, etc., especially in old cultures with no agitation. It is recognized, of course, that agitation increases the oxygen tension in the medium with consequently more positive values. It is, likewise, realized that under conditions of agitation the metab-

olism of an organism may vary in certain respects from what would be termed normal, for it has been reported that agitation tends to decrease cell size after the early logarithmic period of growth (Mudge and Smith, 1933).

In a poorly-poised medium various types of platinum electrodes gave widely different results under identical conditions. There was no great deviation when these same types were used in a poised medium and none when tested in a simple inorganic redox system. Platinum electrodes were used almost exclusively, although a few gold ones were tried from time to time with no resulting obvious advantage. There was no apparent sluggishness of platinum types as compared with gold forms. In regard to this latter point Elema *et al.* (1934), Longsworth and MacInnes (1936) and others have reported the gold electrode to be more sensitive than platinum. This probably depends to some extent upon the type, as well as the amount, of electromotively active materials present in solution. An oxygen electrode effect would seem to account for the difference in values obtained with platinum electrode types.

It is shown that in order to obtain regular values during the growth of organisms in synthetic media under aerobic conditions, some oxidation-reduction system must be introduced which will act as an intermediate between the organisms or their products and the electrodes. This introduces a problem of some complexity for the proper substance must be chosen according to its E'_0 value and its toxicity. The amount added is also of prime importance since an excess may easily poise the potential in one region and thus give no indication as to what is actually happening as the organisms grow. Any one mediator can be good for only a limited Eh range. In regard to the latter point Kluyver and Hoogerheide (1936) have suggested the use of a mixture of oxidation-reduction dyes. There are many who would question the use of dyes and other compounds from the standpoint of toxicity but if an organism can be shown to be unaffected in growth or morphology by such substance there should be no valid objection. Undoubtedly the better values that can be obtained aerobically by the use of nutrient broth media owe their

superiority to the presence of one or more unknown substances that are acting in the same position as these known redox materials.

Anaerobically, in a poorly poised medium, very good agreement was obtained during the first seven hours of growth. At the end of this period there were large numbers of organisms as judged by turbidity. There was then a sudden tendency toward negative values and irregular results were again obtained. The rapid change in potential may be the result of a rapid pH change as suggested by Gillespie and Rettger (1936) but it is hardly reasonable to believe that such a change would occur almost instantaneously. Longsworth and MacInnes (1936) have noted that shifts occurred even when the pH was kept constant, but in no case were they as rapid as those noted here. The electrode phenomena encountered at this time are reproducible with regard to time and are apparently the result of changes occurring so rapidly that the inert electrodes are unable to establish an equilibrium. The almost concordant values, regardless of electrode types, obtained in sterile control culture vessels offer direct evidence against oxygen as an interfering agent in aerobic determinations of this type.

The fact that this bacterial oxidation-reduction system is oxygen labile (and certainly many, if not all, such bacterial systems would fall into this category), introduces another very important aspect into its measurement. How can it be said that a given organism establishes a characteristic potential in any medium if those systems responsible for the potential are exposed to the air previous to, and at the time of, measurement? The value obtained may stand the test of repetition by the initial investigator but it certainly has no absolute significance since it is dependent upon a number of factors over which no control has been exerted. An anaerobic or closed system would seem to be of prime importance.

pH changes have been thought by some investigators to be the source of some irregularities. The end pH value in the broth medium was in the alkaline range of pH 7.2 and that of the synthetic medium was in the acid range of pH 4.5. This latter

value was obtained in both poorly poised and semi-poised media. Hence, it is difficult to reconcile the irregularities as being due solely to pH changes during growth. There is, as yet, a lack of sufficient knowledge of the oxidation-reduction systems involved to warrant an attempt at making corrections for pH changes.

Many factors undoubtedly play important rôles in time-potential relationships of bacterial cultures. The data presented in this paper bring forth certain neglected aspects of the problems involved in the measurement of the apparent oxidation-reduction potentials established by bacteria when growing in synthetic media.

SUMMARY

In this study it is shown that a number of factors, neglected by other workers, are of importance in the measurement of apparent oxidation-reduction potentials in bacterial cultures.

The accuracy of results obtained from broth and synthetic medium cultures of *Escherichia coli* are compared. It is demonstrated that more comparable results are obtained in the broth medium. This fact is apparently due to the inherent presence of poising materials in the broth medium for when such materials are added to the synthetic medium, comparable results are likewise obtained.

The necessity for controlling the accessibility of oxygen to the culture medium is considered to be a factor influencing the magnitude of time-potential values. This is true because many if not all these redox systems are oxygen labile. Oxygen would, in such cases, affect the ratio, Ox./Red., upon which the potential determinations are dependent.

It is shown that zone effects, which are considered to result from temporary poisoning of the electrodes with bacteria or gas bubbles, account for slight variations. Agitation of the culture medium obviates these difficulties.

The type of bright platinum electrode used is only a factor for consideration when determinations in poorly poised media

are made in the presence of air. Under such conditions oxygen electrode effects are probably encountered.

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SOME EFFECTS OF ASSOCIATION AND COMPETITION ON ACETOBACTER

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I. INTRODUCTION

It is well known that one microorganism may inhibit or stimulate the growth and activity of another when the two are grown in association. Although such phenomena were early recognized, the nature of this effect is not definitely known in many instances. This competition or association may be due to the influence of the microorganisms themselves upon one another; to the production of metabolic products; to the effect of substrates or to other factors. The general aspects of association of microorganisms appear in reviews of the literature by DeBary, 1879; Ward, 1899; Lafar, 1904–07; Kruse, 1910; and more recently by Buchanan and Fulmer, 1930; Gause, 1934; Waksman, 1937; Porter and Carter, 1938 and others.

The competition between acetic-acid bacteria and yeasts in fruit juices and other fermentable solutions, which results in rapid acetification and inhibition of alcoholic fermentation, is an example of the general phenomenon. Although this competition has been widely recognized (Lafar, 1904; Kruse, 1910; Cruess, 1912; Lamb and Wilson, 1923; Buchanan and Fulmer, 1930 and others) the factors influencing rapid acetification, such as the species of yeasts and acetic-acid bacteria, the relative populations of the competing organisms, the chemical and physical conditions of the substrate are not well known. Further general observations on this type of association are presented in this paper.

II. EXPERIMENTAL

A. Source of cultures

Twenty-seven strains of *Acetobacter* isolated from commercial samples of Muscat grape musts (*Vitis vinifera* var. Muscat of Alexandria) in which the alcoholic fermentation was inhibited in its initial stages by rapid acetification were studied. These strains closely resemble *Acetobacter aceti* as defined by Visser 'T Hooft, 1925.

The ability of the cultures to attack sugars, glucosides, alcohols and the salts of organic acids was determined by inoculation into a basal medium containing 0.5 per cent tryptone (Difco), 0.2 per cent yeast-extract powder (Difco) and 0.1 per cent K_2HPO_4 in the presence of the substance to be tested. The medium was adjusted to pH 6.8–7.0 before sterilization at 15 pounds pressure for 15 minutes. Abundant growth and change in the reaction of the medium were taken as criteria of the utilization of the compounds tested. The distinguishing biochemical characters of the cultures are shown in table 1.

Glucose was the only one of the sugars or glucosides tested which was utilized. No production of calcium oxy-gluconate was noted even after 1 month. All strains produced an off-flavor known as "mousiness" when grown in sterile grape juice or grape concentrate media. The production of this characteristic "mousey" off-flavor heretofore has apparently been noted only as a characteristic of bacteria of the genus *Lactobacillus* associated with the spoiling of wines. (Müller-Thurgau and Osterwalder, 1913; Douglas and Cruess, 1936, and Arena, 1936, and others.)

The following known species of yeasts and bacteria were also used:

Saccharomyces ellipsoideus, strains No. 66, "Burgundy" and "Champagne"; *Hansenula* (*Willia*) *anomala*, American Type Culture No. 2577; *Hansenula* (*Willia*) *saturnus*, American Type Culture No. 2579; *Zygosaccharomyces priorianus* from Dr. H. H. Hall of the U.S.D.A. and *Schizosaccharomyces octosporus* from Dr. C. B. van Niel of the Hopkins Marine Station, all from the Division of Fruit Products Culture Collection.

Acetobacter aceti 4920, 4969, *A. pasteurianum* 6033, *A. xylinum* 4939, *A. suboxydans* 621, and *A. peroxydans* 838 were obtained from the American Type Culture Collection; *A. acetic* 612, *A. pasteurianum* 613, *A. xylinum* 1375, *A. acetosum* 2224, *A. suboxydans* 3734 and *A. keutzingianum* 3924 from the National

TABLE 1

Biochemical characters of the 27 Acetobacter cultures

CHARACTER	INCUBATION PERIOD	
	2 days	7 days
	Number of cultures giving positive reactions	
Catalase*	27	27
Growth in Hoyer's solution†	Apparent after 2 weeks incubation	
Nitrate reduction*	‡	18
Acid production from sugars:*		
Glucose	0	27
Acid production from alcohols:†		
Ethyl	27	27
Propyl	0	27
Decomposition of organic acid salts:*		
Acetic	0	23
Malonic	22	26
Malic	26	27
Citric	0	11
Tartaric	24	27
"Mousey" off-flavor in grape juice media and musts:*	‡	27 (5 to 7 days)
Brown pigment on wort agar slants*	Not observed in 1 month	

* Incubation at 37°C.
† Incubation at 30°C.
‡ Not determined.

Collection of Type Cultures and *A. melangenum* (Kluyver strain) from Dr. C. B. van Niel.

Lactobacillus pentoaceticus, *L. mannitopeus* and *L. gayonii* obtained from the University of Wisconsin; *L. lycopersici*, *L. fructovorans* and *L. gracilis* from Dr. C. S. Pederson[1] and a species, tentatively recognized as *L. kilgardii*, from the Division of Fruit Products Collection were used.

[1] For the most recent view concerning the classification of these species see Pederson (1938).

B. Observations on associative growth and rapid acetification

Grape concentrate (70°–80° Balling) was diluted with water to make a medium having a sugar content of approximately 20 per cent (20° Balling). This medium was dispensed in 75–80 cc. portions in 130 cc. bottles, which were then plugged with cotton and sterilized. It contained 0.012 gram volatile acid calculated as acetic and 0.300 gram total acid calculated as tartaric in 100 cc., determined by the methods of the Association of Official Agricultural Chemists (1930).

A 1 cc. inoculum of yeast culture 2 to 3 days old from beer-wort broth and one 2 mm. loopful of bacterial culture from liver infusion or wort-agar slants were used singly or in association as indicated in the following experiments.

a. Association of Acetobacter strains and yeasts. The associative action between the *Acetobacter* cultures and the various species of yeasts was determined by the volatile acid produced (table 2). It will be noted that marked volatile acid production occurred when the *Saccharomyces ellipsoideus* strains were grown in association with the *Acetobacter* cultures and was especially high with the "Champagne" strain. A lesser amount of volatile acid was observed with the cultures of *Zygosaccharomyces priorianus* and *Schizosaccharomyces octosporus*. With *Hansenula anomala* and *Hansenula saturnus* even less acetification occurred.

At the incubation temperature of 37°C. fermentative activity of the yeasts used in these studies is markedly below that at their optimum temperature of approximately 27°C. This factor is in part responsible for the rapid acetification noted when the various yeasts and bacteria were grown in association. The differences noted between the cultures of *Zygosaccharomyces priorianus, Schizosaccharomyces octosporus, Hansenula anomala* and *Hansenula saturnus* and those of *Saccharomyces ellipsoideus* are probably due to the lower rates at which the former ferment sugar as well as to differences in the nature of the intermediate products and the optimum temperatures for growth and activity.

b. Effect of incubation temperature on associative growth and acetification. In commercial practice rapid acetification of fer-

menting musts is favored by high temperatures and the cessation of yeast fermentation is generally due to the effect of temperature as well as the high acetic acid content. When naturally fermenting musts are properly cooled during the process of fermen-

TABLE 2

*Volatile acid production by 27 Acetobacter strains grown in association with various yeasts**

VOLATILE ACID PRODUCED (AS ACETIC)	NUMBER OF ACETOBACTER CULTURES PRODUCING VOLATILE ACID IN PRESENCE OF:						
	S. ellipsoideus			*H. anomala*	*H. saturnus*	*Z. priorianus*	*S. octosporus*
	66	"Burgundy"	"Champagne"				
grams/100 cc.							
0–0.099	1			8	27	3	7
0.100–0.199	4	4	1	12			5
0.200–0.299	1	1	2	7		3	1
0.300–0.399	2	5				3	4
0.400–0.499	6	3				10	4
0.500–0.599	4	4				4	4
0.600–0.699	4	3				3	2
0.700–0.799	3	1	3			1	
0.800–0.899	2		1				
0.900–0.999		2	3				
1.000–1.099		1					
1.100–1.199		3	2				
1.200–1.299			1				
1.300–1.399			3				
1.400–1.499			2				
1.500–1.599			3				
1.600–1.699			1				
1.700–1.799							
1.800–1.899							
1.900–1.999			3				
2.000–2.099			1				
2.100–2.199			1				

* Incubation at 37°C. for 3 days.

tation, the production of volatile acid is usually very slight. Therefore it was thought likely that the temperature had a marked effect on acetification in the associative action of yeasts and the *Acetobacter* cultures. To determine the effect of tem-

perature on volatile acid production *Saccharomyces ellipsoideus* No. 66 was grown in association with each of the 27 *Acetobacter*

TABLE 3

Effect of temperature of incubation on acetification

YEAST 66 WITH ACETOBACTER NUMBER	TEMPERATURE*			
	25°C.	31°C.	37°C.	42°C.
	Volatile acid—grams/100 cc. as acetic			
68	0.360	0.465	0.741	0.239
69	0.288	0.429	0.771	0.192
73	0.279	0.252	0.571	0.114
83	0.562	0.321	0.546	0.184
84	0.233	0.498	0.551	0.110
85	0.258	0.465	0.609	0.140
86	0.145	0.270	0.402	0.158
88	0.203	0.543	1.164	0.176
90	0.204	0.498	0.648	0.098
92	0.180	0.186	0.300	0.260
98	0.189	0.477	0.903	0.286
99	0.165	0.228	0.468	0.256
100	0.621	0.588	0.780	0.238
101	0.276	0.183	0.633	0.246
102	0.147	0.213	0.537	0.180
108	0.180	0.282	0.405	0.036
109	0.102	0.306	0.477	0.268
110	0.136	0.117	0.171	0.175
111	0.099	0.303	0.591	0.162
112	0.153	0.330	0.618	0.138
113	0.153	0.149	0.606	0.535
114	0.183	0.333	0.483	0.480
115	0.150	0.172	0.378	0.220
116	0.297	0.177	0.516	0.225
117	0.165	0.306	0.510	0.326
118	0.168	0.168	0.450	0.381
119	0.105	0.180	0.324	0.305
A.T.C.C. 4920†		0.180	0.068	
No. 66 alone	0.050	0.054	0.069	0.021
Blank	0.012	0.012	0.012	0.012

* Incubation period of 3 days.

† Volatile acid not produced by other type *Acetobacter* strains.

strains at 25°, 31°, 37°, and 42°C. for a period of 3 days. Inoculations were made in quadruplicate and one of each combined culture was incubated at each temperature (table 3). Known

Acetobacter species were also grown in association with yeast No. 66 at 31° and 37°C. Under the conditions of these experiments incubation at 37°C. resulted in the highest volatile acid production by the Muscat strains of *Acetobacter*. It is of interest that none of the known species of *Acetobacter* produced appreciable amounts of volatile acid when grown with the yeast at an incuba-

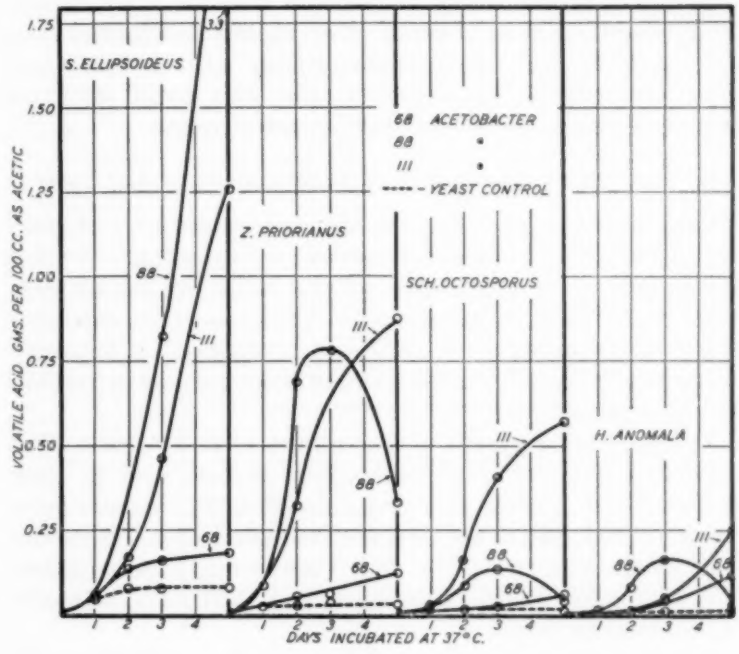

FIG. 1

tion temperature of 37°C. and only one strain, *Acetobacter aceti* A.T.C.C. 4920, was capable of doing so at 31°C.

c. Observations on rate of acetification. The rate of acetification caused by the 27 *Acetobacter* strains grown with various yeasts was found to depend upon the type of yeast and strain of bacterium. Furthermore a constant difference in the total volatile acid produced as a result of this association was observed when cultures were incubated for a given period of time.

The rates of acetification produced by the *Acetobacter* cultures in the presence of *Saccharomyces ellipsoideus* No. 66, *Zygosaccharomyces priorianus*, *Schizosaccharomyces octosporus* and *Hansenula anomala* were determined from the volatile acid produced after 1, 2, 3 and 5 days incubation at 37°C. Typical results are shown in figure 1.

The rates of acetification varied both with the strain of bacterium and the species of yeast. Two typical acetification rates were observed with the bacteria growing with *Saccharomyces ellipsoideus* whereas three distinct rates were noted when the bacteria were grown with the other species of yeasts.

C. Observations of association of Lactobacillus and Acetobacter

Bacteria of the genus *Lactobacillus* are frequently found growing with strains of yeast and *Acetobacter* in fermenting fruit juices undergoing rapid acetification. Under these conditions competitive growth is to be expected. Therefore the competitive effects of association on volatile acid production by heterofermentative strains of *Lactobacillus* and *Saccharomyces ellipsoideus* No. 66 were studied.

To insure rapid growth of the cultures of *Lactobacillus* when inoculated into the grape concentrate medium used in these studies, a 1 cc. portion of young liver infusion or tomato juice-tryptone broth culture was used as an inoculum when the bacteria were grown with yeast No. 66. Volatile acid determinations were made after 3 and 5 days incubation at 37°C. The results are shown in table 4.

It will be noted that all of the strains of *Lactobacillus*, with the exception of *L. fructovorans* and *L. gracilis*, produced significant quantities of volatile acid after 3 days incubation. *L. fructovorans* did not produce a large quantity of volatile acid even after incubation for 5 days, although on longer incubation the amount of volatile acid materially increased. A definite decrease in the amount of volatile acid formed was noted when the species of *Lactobacillus* were grown in association with *Saccharomyces ellipsoideus* No. 66.

Volatile acids were also produced when the *Acetobacter* strains were grown in the presence of these same lactobacilli. Although *Acetobacter* strains 68 and 111 did not significantly influence the

TABLE 4

Effect of association on volatile acid production by species of Lactobacillus

ORGANISMS	BACTERIA ALONE		BACTERIA WITH YEAST NO. 66	
	Volatile acid production (grams per 100 cc. as acetic)			
	3 days*	5 days	3 days	5 days
L. mannitopeus	0.244	0.350	0.108	0.120
L. gayonii	0.180	0.176	0.144	0.168
L. pentoaceticus	0.115	0.160	0.120	0.120
L. lycopersici	0.344	0.448	0.170	0.204
L. fructovorans	0.014	0.029	0.077	0.092
L. gracilis	0.067	0.196	0.084	0.105
L. hilgardii	0.184	0.298	0.091	0.104
S. ellipsoideus (No. 66 alone)			0.076	0.071
Uninoculated control	0.009	0.011	0.012	0.012

* Incubation at 37°C.

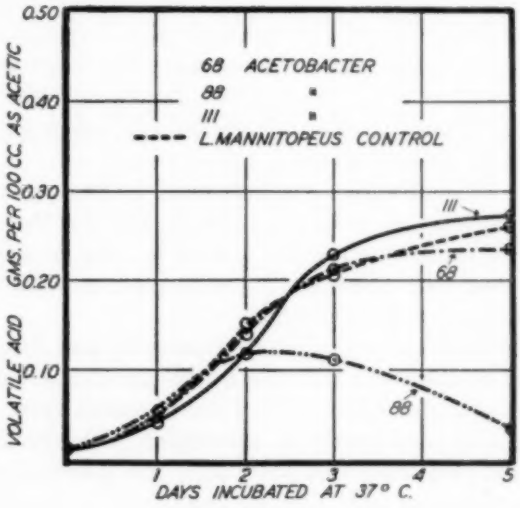

FIG. 2

amount of volatile acid produced by the species of *Lactobacillus*, *Acetobacter* 88 caused a definite decrease in volatile acid. The curves in figure 2 showing the rates of acetification with *Lactobacillus mannitopeus* grown in association with various *Acetobacter* strains are typical of the differences in acetification found for these competing bacteria. A comparison of the curves in figure 1 and figure 2 indicates a striking similarity between those observed when certain of the yeasts were grown in association with *Acetobacter* 88 and the one noted when *L. mannitopeus* was grown with the same acetic bacterium. The observations suggest that the differences in behavior of these *Acetobacter* strains is due, in part at least, to differences in their oxidative activity.

III. SUMMARY

Acetobacter cultures which seem to be similar in their characteristics from the standpoint of detailed laboratory study react differently when grown in association with other organisms. The power of rapid acetification is not possessed by all strains of *Acetobacter* when grown in association with yeasts. The bacteria isolated from "stuck" wines all possessed this characteristic whereas well-known strains obtained from various collections were unable to bring about rapid acetification when grown with yeast at 37°C. One known strain, *Acetobacter aceti* A.T.C.C. 4920 caused a small amount of acetification when grown in association with yeast at 30°C.

The rate of acetification was influenced by the types of yeasts grown in association with the bacteria and by the strains of bacteria. Several characteristic trends in volatile acid production were observed. The rate of acetification was also influenced by the temperature of incubation.

Competition between strains of *Lactobacillus* and *Saccharomyces ellipsoideus* resulted in a decrease in the amount of volatile acid formed in 5 days at 37°C. The rates of acetification by *Lactobacillus mannitopeus* grown in association with the *Acetobacter* strains paralleled those obtained with the *Acetobacter* cultures grown in association with different yeasts.

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HEAT STERILISED REDUCING SUGARS AND THEIR EFFECTS ON THE THERMAL RESISTANCE OF BACTERIA

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That the thermal resistance of at least some types of microorganisms is increased when heated in hypertonic solutions of various carbohydrates is now well established. A review of the literature concerning this subject has been presented by Fay (1934). This author found that sucrose and glucose in concentrations above 0.75 M were protective for *Escherichia coli*. No protection was obtained with saturated lactose solution and 50 per cent maltose solution gave only slight protection. In these experiments the bacteria were heated in nutrient broth solutions of the sugars. It was not stated by what means the solutions were sterilised.

In a later paper Baumgartner and Wallace (1934) published results which were not in agreement with Fay's findings in respect to lactose. They found that lactose in 17 per cent solution and sucrose, glucose and invert sugar in 20 per cent solutions were each protective for *Escherichia coli*. In this investigation the sugars were sterilised by autoclaving in a dry state and then dissolved in sterile buffer solution.

The effects of heat sterilisation on media containing sugars have been investigated by several workers. Mudge (1917) reported that hydrolysis of lactose and maltose was greater when sterilised in streaming steam for three successive days than when once autoclaved at 15 pounds pressure for 15 minutes. Fulmer, Williams and Werkman (1931) noted growth stimulation of yeast and certain bacterial species including *Escherichia coli* in a medium (containing 0.6 gram NH_4Cl, 0.2 gram K_2HPO_4 and 5.0 grams

glucose per 100 cc.) which had been sterilised at 15 pounds pressure for 15 minutes. The stimulant was not caramel but a substance produced parallel with caramel. Previously, Lewis (1930) reported that growth of some types of bacteria was completely inhibited in media containing either glucose, maltose, lactose, galactose or levulose which together with phosphate and a source of nitrogen had been sterilised at 122°C. for 15 minutes. The reaction depended on the concentration of sugar, phosphate and nitrogen compound present and it was concluded that the inhibition was due to the conversion of the nitrogen compound into a form not suitable for assimilation by certain bacterial species.

In the course of work in this laboratory on the quantitative effects of sugars on the thermal resistance of *Escherichia coli* it was found that the method of sterilising *reducing* sugars greatly influenced the results obtained. The experiments indicated that for sugars of this type it is necessary to adopt the filtration method of sterilisation, since their heat treatment in buffer solution or nutrient broth results in the formation of material which reduces bacterial heat resistance. The toxic material was formed during heat sterilisation of the sugars in solutions with or without nitrogenous compounds. The material acts as a direct toxic agent and is not connected with alteration of nutrient materials.

METHODS

The sugars used in this work were supplied by British Drug Houses Ltd., and were the purest available. They were dissolved in McIlvaine's disodium phosphate-citric acid buffer solution or nutrient broth (both at pH 7.0). The basic thermal resistance of the test organism was estimated in plain buffer solution or nutrient broth. A suspension of a filtered, 24 hour agar growth of *Escherichia coli*[1] was suitably diluted and from a pipette graduated in 0.01 cc., exactly 0.25 cc. was mixed with 20 cc. amounts of the various solutions. These were subjected to a 1-minute uniform shaking. The control tube (without sugar) was diluted 1:1000 with sterile distilled water and then plated

[1] National Collection of Type Cultures, strain No. 86.

in duplicate. Counts were not made of the sugar solutions, equal inoculation being assumed in each instance. Three cubic centimeters amounts of each inoculated solution were then placed into sterile tubes (5 x ½-inch) and these were heated in a water bath at 54°C. for eight minutes. No differences in heat penetration into the various solutions were noted. After rapid cooling the solutions were diluted 1:100 and 1 cc. amounts of this and also the undiluted solution were plated in duplicate into 20 cc. of plain nutrient agar (pH 7.0). The amount of sugar added to the test plates via the inoculum was, in the case of the 1:100 dilution of glucose, only 0.0045 per cent, an amount which was considered unlikely to influence the results appreciably. In the plates receiving undiluted solution the concentration of glucose reached 0.45 per cent, but where such plates were used in obtaining counts the number of survivors was so low that any stimulative influence of the sugar was disregarded. The plates were incubated for 3 days at 37°C., counted and held for a further period of 4–5 days at room temperature. Counts exceeding 10,000 per cc. are given to the nearest 500.

EXPERIMENTAL

1. The effects of heat-sterilised and filter-sterilised sugar solutions on the thermal resistance of E. coli

The sugars were used in 0.5 M concentration. Two series of sugar solutions were tested in which the solvents were buffer solution and ordinary nutrient broth (pH 7.0). The solutions were sterilised at 112°C. for 15 minutes. Due to this treatment caramelisation and acid formation occurred to a varying degree in each of the reducing sugar solutions. After sterilising, the pH values of the solutions were adjusted to 7.0 with sterile N/10 NaOH solution.

Table 1 gives results with buffered solutions and broth solutions of the sugars. These form two separate experiments but are included in one table for convenience of presentation.

The results showed that in each instance, autoclaved solutions of reducing sugars gave an increased lethal effect. This was also apparent when fractionally sterilised sugar solutions were used

(table 2). In this case, sugar solutions in buffer were sterilised in streaming steam for 30 minutes on 3 successive days. Changes in pH which occurred in the reducing sugar solutions were adjusted after sterilising.

These effects, in which heat sterilised reducing sugar solutions increased the thermal destruction of *E. coli*, did not occur if the solutions were sterilised by filtration. With this method of

TABLE 1

Survival of E. coli exposed for 8 minutes at 54°C. in autoclaved sugar solutions

HEATING MEDIUM	NUMBER OF VIABLE CELLS PER CC.	
	Before heating	After heating
Plain buffer (control)	232,000	3,200
0.5 M Glucose in buffer	232,000	184
0.5 M Galactose in buffer	232,000	104
0.5 M Lactose in buffer	232,000	210
0.5 M Maltose in buffer	232,000	0
0.5 M Sucrose in buffer	232,000	21,500
0.5 M Mannitol in buffer	232,000	10,500
0.5 M Glycerol in buffer	232,000	7,500
Plain broth (control)	224,000	16,000
0.5 M Glucose in broth	224,000	6,000
0.5 M Galactose in broth	224,000	4,200
0.5 M Lactose in broth	224,000	3,500
0.5 M Maltose in broth	224,000	7,000
0.5 M Sucrose in broth	224,000	33,500
0.5 M Mannitol in broth	224,000	24,000
0.5 M Glycerol in broth	224,000	21,000

sterilisation, all the sugars tested increased the number of cells surviving heat treatment (see table 3). In this experiment a Seitz Filter fitted with Seitz E. K. filter pads was used. Heat treatment of the solutions was completely avoided.

Lewis (1930) observed that solutions of glucose in distilled water autoclaved at 122°C. did not cause growth inhibition when incorporated in a nutrient medium.

We have noted that for the production of toxic material from solutions of glucose in distilled water, it was necessary to heat the solution until caramelisation occurred. This requires treatment

TABLE 2

Survival of E. coli exposed for 8 minutes at 54°C. in fractionally-sterilized sugar solutions

HEATING MEDIUM	NUMBER OF VIABLE CELLS PER CC.	
	Before heating	After heating
Plain buffer (control)	420,000	8,500
0.5 M Glucose in buffer	420,000	2,100
0.5 M Galactose in buffer	420,000	120
0.5 M Lactose in buffer	420,000	30
0.5 M Maltose in buffer	420,000	256
0.5 M Sucrose in buffer	420,000	25,000
0.5 M Mannitol in buffer	420,000	21,000
0.5 M Glycerol in buffer	420,000	18,500

TABLE 3

Survival of E. coli exposed for 8 minutes at 54°C. in filter-sterilized sugar solutions

HEATING MEDIUM	NUMBER OF VIABLE CELLS PER CC.	
	Before heating	After heating
Plain buffer (control)	272,000	5,100
0.5 M Glucose in buffer	272,000	14,500
0.5 M Galactose in buffer	272,000	11,500
0.5 M Lactose in buffer	272,000	49,000
0.5 M Maltose in buffer	272,000	13,500
0.5 M Sucrose in buffer	272,000	32,500
0.5 M Mannitol in buffer	272,000	15,000
0.5 M Glycerol in buffer	272,000	10,000
Plain broth (control)	280,000	11,000
0.5 M Glucose in broth	280,000	22,000
0.5 M Galactose in broth	280,000	22,500
0.5 M Lactose in broth	280,000	33,000
0.5 M Maltose in broth	280,000	20,500
0.5 M Sucrose in broth	280,000	28,000
0.5 M Mannitol in broth	280,000	25,000
0.5 M Glycerol in broth	280,000	18,500

far in excess of that necessary when buffer or broth is used. Heat treatment of the same order as applied to buffered or broth solutions does not produce caramelisation in distilled water solutions of glucose and solutions so treated are not toxic for *E. coli* at

54°C. On the other hand, if distilled water solutions of glucose are heated until caramel is formed, they increase the rate of thermal destruction of the organism (table 4). The solutions of glucose in distilled water were heated in large quartz tubes. In the case of the solution autoclaved for 15 minutes at 112°C., no change in colour occurred but the solution heated for 3 hours

TABLE 4

Survival of E. coli exposed for 8 minutes at 54°C. in distilled water-treated glucose solutions

HEATING MEDIUM	TREATMENT	NUMBER OF VIABLE CELLS PER CC.	
		Before heating	After heating
1. Plain buffer (control)		254,000	4,500
2. 0.5 M Glucose in buffer	Sterilised at 112°C. for 15 minutes	254,000	704
3. 0.5 M Glucose in buffer	Filter-sterilised	254,000	12,500
4. 0.5 M Glucose in buffer	Heated in distilled water at 112°C. for 15 minutes; evaporated and redissolved in buffer. Solution then filter-sterilised	254,000	9,800
5. 0.5 M Glucose in buffer	Heated in distilled water at 130°C. for 3 hours; evaporated and redissolved in buffer. Solution then filter-sterilised	254,000	1,100
6. Plain buffer	Prepared with distillate from evaporation process of distilled water solution of dextrose in 4	254,000	5,800
7. Plain buffer	Prepared with distillate from evaporation process of distilled water solution of dextrose in 5	254,000	4,000

at 130°C. became straw coloured and acid was formed. The solutions were evaporated *in vacuo* (210 mm. Hg) until the sugar remained as a very thick syrup (about 80 per cent glucose). The sugar tended to char if evaporated beyond this point. The syrup was then redissolved in buffer solution to give 0.5 M concentration of glucose. The solutions were finally sterilised by filtration. The distillates obtained from the evaporation proc-

esses were used for preparing plain buffer solutions and were included in the tests with other controls.

Heat sterilisation of glucose without production of toxic material is possible if the sugar is autoclaved in a dry state and subsequently dissolved in cold sterile buffer solution. For example, in plain buffer solution, with an initial concentration of 210,000 viable cells per cubic centimeter, 4,500 cells per cubic centimeter survived heating at 54°C. for 8 minutes. In 0.5 M glucose (sugar sterilised dry for 15 minutes at 112°C. and then dissolved in cold sterile buffer) the survivors numbered 10,500 per cubic centimeter.

TABLE 5

Survival of E. coli exposed for 8 minutes at 54°C. in autoclaved dextrose solutions (2.5 M and 0.5 M concentration)

SOLUTION	TREATMENT	NUMBER OF VIABLE CELLS PER CC.	
		Before heating	After heating
1. Plain buffer	Autoclaved	560,000	6,500
2. 2.5 M Glucose in buffer	Autoclaved	560,000	350
3. 2.5 M Glucose in buffer	Filter-sterilised	560,000	120,000
4. 0.5 M Glucose in buffer	Autoclaved	560,000	720
5. 0.5 M Glucose in buffer	Filter-sterilised	560,000	26,500

Experiment did not support the hypothesis that, with more concentrated sugar solutions, the effect of toxic substances resulting from heat sterilisation would be masked by the protective effect of the increased amount of sugar. The solutions used were 2.5 M and 0.5 M glucose in buffer (pH 7.0). These were autoclaved at 112°C. for 15 minutes. It was noted that the 2.5 M solution was caramelised to a greater extent than the 0.5 M solution by this treatment, the former was deep straw, whilst the latter was pale straw in colour. Acid formed during sterilisation was afterwards neutralised with sterile NaOH solution. Controls consisted of duplicate samples, filter sterilised, of the sugar solutions and plain buffer solution.

2. The production of caramel and toxic material

Results of the experiments with distilled water solutions of glucose suggested that caramelisation is a necessary complement to the production of toxic material. If this is so, then increased caramelisation of a solution would be expected to lead to increased toxicity.

To test this theory the following experiment was made. Twenty cubic centimeter amounts of 0.5 M glucose in buffer solution (pH 7.0) were autoclaved in large test tubes, the treatment varying with each tube. A further 20 cc. of the same solution were filter-sterilised, as were 20 cc. of plain buffer solution. In the heated solutions caramelisation increased according to the severity of the heat treatment. Acid production occurred and adjustments to pH 7.0 were made with sterile NaOH solution. Results are shown in table 6.

The data given in table 6 indicate that the toxic material arising from the heat treatment of dextrose solution is produced parallel with caramel or that the caramel is in itself toxic.

It was found that the toxicity of a caramelised, buffered glucose solution was only slightly reduced by treatment with a purified adsorbing charcoal (table 7). In this experiment, the glucose solution (0.5 M in buffer) was autoclaved for 20 minutes at 120°C. This treatment caused the solution to become dark brown in colour and acid was formed. After treatment with charcoal for 60 minutes at 37°C. the solution was passed through a Seitz filter. The solution was then only faintly coloured, practically all the caramel having been removed. After filter-sterilising the solution was neutralised with sterile NaOH solution.

The data given in table 7 indicate that almost complete removal of caramel from a heated glucose solution has only a very small effect on the toxic strength of the solution. It would thus appear that caramel is not the toxic agent. The slight decrease in toxicity which occurred upon removal of caramel was possibly due to adsorption of a small amount of the toxic material simultaneously with caramel.

TABLE 6

Survival of E. coli exposed for 8 minutes at 54°C. in dextrose solutions caramelised to a varying degree

SOLUTION	TREATMENT	COLOUR (INDICATING EXTENT OF CARAMELISATION)	NUMBER OF VIABLE CELLS PER CC.	
			Before heating	After heating
1. Plain buffer	Filter-sterilised		360,000	4,500
2. 0.5 M Glucose in buffer	Filter-sterilised		360,000	24,000
3. 0.5 M Glucose in buffer	1 minute at 112°C.	No change (as in 1 and 2)	360,000	7,500
4. 0.5 M Glucose in buffer	15 minutes at 112°C.	Pale straw	360,000	1,400
5. 0.5 M Glucose in buffer	60 minutes at 112°C.	Deep straw	360,000	0
6. 0.5 M Glucose in buffer	60 minutes at 120°C.	Dark red-brown	360,000	0

TABLE 7

Survival of E. coli exposed for 8 minutes at 54°C. in heated dextrose solutions before and after adsorption with charcoal

SOLUTION	TREATMENT	NUMBER OF VIABLE CELLS PER CC.	
		Before heating	After heating
1. Plain buffer (control).....	Filter-sterilised	420,000	3,800
2. 0.5 M Glucose in buffer.....	Autoclaved 20 minutes at 120°C.	420,000	0
3. 0.5 M Glucose in buffer.....	Autoclaved for 20 minutes 120°C.; treated with charcoal then filter-sterilised	420,000	120
4. 0.5 M Glucose in buffer.....	Not heated, treated with charcoal, then filter-sterilised	420,000	23,000

In connection with the adsorption of caramel, it may be stated that colorimetric tests failed to reveal any marked removal of caramel from a heated glucose solution after treatment with massive doses of washed cells of *E. coli*.

3. Effects of heat-sterilised glucose broth on the growth of E. coli

In addition to the toxicity of heat-sterilised glucose nutrient broth demonstrated in the thermal resistance tests (table 1), it has been found that such a medium is also capable of retarding the growth of *E. coli* (table 8). Lewis (1930) found that the production of inhibitory media was affected by the phosphate content of the medium, at least 0.2 per cent K_2HPO_4 being required for inhibition to occur. He also found that a glucose medium which was inhibitory for some species did not inhibit the growth of *E. coli*. It would seem, however, that Lewis's

TABLE 8

The growth of E. coli in heat-sterilised and filter-sterilised glucose-nutrient-broth

MEDIUM	TREATMENT	TIME FOR GROWTH TO BECOME MACROSCOPICALLY VISIBLE	GROWTH AFTER 24 HOURS	GROWTH AFTER 7 DAYS
		hours		
1. 0.5 M Glucose broth.....	Autoclaved	5½	+	+++
2. 0.5 M Glucose broth.....	Filter-sterilised	4	++	++++
3. 0.5 M Glucose broth, 0.5 per cent K_2HPO_4.....	Autoclaved	6½	∓	+++
4. 0.5 M Glucose broth, 0.5 per cent K_2HPO_4.....	Filter-sterilised	4	++	++++

++++ = maximum growth.

experiments were concerned with complete inhibition rather than the slight effects considered in the present report.

In the heated glucose nutrient-broth which we have noted to be toxic at 54°C. and also slightly inhibitory to the growth of *E. coli* at 37°C., the only phosphate present was that occurring normally in the medium, about 0.06 per cent calculated as K_2HPO_4. Experiments were made in which the phosphate content of the medium was increased by addition of K_2HPO_4. The heated media were sterilised at 112°C. for 15 minutes. Changes in pH occurred during sterilisation, and the media showed signs of caramelisation; that containing added phosphate being more

affected than that without added phosphate. After sterilising, the pH of the heated media was adjusted to the original value of 7.0 with sterile NaOH solution. The control media (pH 7.0) were sterilised by filtration. To 20 cc. of each of the media prepared, was added one drop of a filtered distilled water suspension of *E. coli* and the tubes incubated at 37°C. Before inoculation all tubes were brought to a temperature of 37°C. by immersion in a water-bath for 20 minutes.

It will be seen that the growth of *E. coli* is delayed in heat-sterilised glucose broth. Increasing the phosphate content of the medium appears to increase the delay. The delay persists throughout the growth of the culture and in view of the increased thermal destruction in similar media (table 1) it is reasonable to suppose that it is due to direct toxic action on the bacteria of a substance produced during sterilisation of the medium. Topley and Wilson (1929), discussing the growth of bacteria in unsuitable media state: "In a medium that is unfavourable—for example one with too high a hydrogen ion concentration—the lag phase is often greatly prolonged; several hours may elapse before the organism begins to grow, but once it has started, it may grow as well as in a medium of optimum reaction. This statement is only true within certain limits; if the medium is very acid, then the growth may not only be slow to begin, but may remain poor in its later stages. A similar observation has been made by Moore (1915) relative to the growth of pneumococci in a medium containing a high dilution of disinfectant."

4. Preliminary chemical investigation of heat-sterilised glucose solutions[2]

A preliminary chemical investigation into the nature of the toxic material has been made. A solution of 0.5 M glucose in buffer solution was autoclaved for 15 minutes at 112°C. which treatment caused caramelisation, rendering the solution straw coloured. This solution was steam distilled from acid solution, but the distillate was negative to tests for acid and aldehyde; the

[2] I am indebted to Dr. H. K. Dean of this laboratory for carrying out this portion of the work.

distillate did not decolorize bromine water and no colouration was obtained on adding $FeCl_3$.

With a similar solution of sugar heavily caramelised, by autoclaving for 3-hours at 120°C., steam distillation from acid solution resulted in an acid distillate in which aldehyde was detected by means of Schiff's reagent. The distillate reduced $HgCl_2$ and decolorized bromine water. Tests for oxalic acid in the original sugar solution and for formic acid in the distillate were negative. On treatment of the distillate with $FeCl_3$ a purplish colouration was obtained which was not phenolic in origin and may have been due to the presence of a keto-enol substance. Pyruvic acid was not present.

A solution of glucose in distilled water, slightly caramelised by heating at 130°C. for 3-hours, gave no precipitate on addition of lead acetate solution.

DISCUSSION

The object of this investigation was to ascertain the effects on the thermal resistance of *E. coli* of buffered and nutrient broth solutions of reducing sugars sterilised by various methods. The data collected indicate that the filtration method of sterilisation should be adopted for such work. Using this method, all the sugars tested, including lactose and maltose, increased the number of cells surviving heat treatment. It thus seems that conflicting reports in the literature concerning the effects of certain sugars on bacterial heat resistance may be due to the use of different methods of sterilising the sugar solutions.

Search of the literature has so far failed to reveal any conclusive account of work which would throw light on the nature of the toxic material formed by the thermal treatment of reducing sugars, in solution (excluding distilled water). The so-called caramel is known to be a highly complex mixture containing colloidal constituents (Clayton, 1932) but caramel as such has been excluded by the necessary tests. It would appear, therefore, that along with caramelisation, some other chemical compound is produced, which in the pure state must be highly toxic to bacteria.

Lewis (1930) discussed the literature concerning the transformations which occur in sugars under the influence of heat and various compounds such as phosphates and caustic alkalies, and concluded that the material causing growth inhibition in his experiments was an aldehyde: This aldehyde combined with the nitrogen source in the medium and rendered it non-assimilable. Ambler and Byall (1935) have reported extensive data on the influence of non-sugars on the caramelisation and inversion of sucrose, drawing attention to the deep-seated and complex isomerisations and degradations involved. Unfortunately, their results cannot be applied to the investigations just described.

Further work is obviously desirable in an attempt to isolate and identify the toxic agent. This should be attempted by working with greater bulk and investigating the fractions obtained by adsorption technique. Removal of colloidal complexes should be first effected, e.g., with alumina cream. If the toxic agent has an affinity for protein it may be expected to reveal this by surface film studies in a Langmuir-Adam trough. It is also necessary to ascertain whether the toxic effect is general for a wide range of bacteria.

SUMMARY

Sterilisation of reducing sugar solutions by autoclaving at 112°C. for 15 minutes or steaming for 30 minutes on three successive days results in the formation of material which is directly toxic for *Escherichia coli* at 54°C. and is capable of delaying the growth of this organism at 37°C.

The material is produced simultaneously with caramel in reducing sugar solutions in which the solvent is McIlvaine's citric acid-phosphate buffer or nutrient broth; for its production in distilled water solution, drastic heat treatment, sufficient to cause caramelisation is required. The toxic material does not occur when buffered or broth solutions of reducing sugars are sterilised by filtration.

Filter-sterilised glucose, galactose, lactose, maltose, sucrose, mannitol, and glycerol in 0.5 M concentration in buffer or broth were all found to protect *Escherichia coli* against thermal destruction.

A preliminary chemical investigation failed to reveal the nature of the toxic material.

I wish to thank Dr. William Clayton for his helpful criticism and advice.

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A MICROORGANISM DECOMPOSING GROUP-SPECIFIC A SUBSTANCES

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The use of bacteria to effect the degradation of serologically active carbohydrates was initiated by Avery and Dubos (1930; see also Dubos and Avery 1931) in studies on the capsular carbohydrate of Pneumococcus Type III; and other microorganisms capable of splitting polysaccharides of bacterial origin have since been found. The present report concerns the isolation and cultural characters of an apparently hitherto undescribed bacterium, having specialized food requirements and not growing on most common culture media, which is able to decompose actively the group-specific substance found in the saliva of human beings of blood group A and in various group A preparations obtained from animal materials, namely horse saliva (Landsteiner 1936) commercial pepsin and gastric mucin (Landsteiner and Chase 1936). From chemical investigations it has become probable that the substances underlying the serological differentiation of human blood groups are of polysaccharide nature (for literature, see Landsteiner 1936, Landsteiner and Chase 1936). Apart from adding to the relatively small number of organisms known to attack "specific" polysaccharides—a property not as common as might have been supposed—the description of a new species with unusual characters may be of interest.

Of bacteria which decompose serologically active complex polysaccharides, mention may be made of the following: the *Myxococcus* of Morgan and Thaysen which acts upon several specific polysaccharides of bacterial origin (Morgan and Thaysen 1933; Meyer and Morgan 1935) and also "group specific" A substances (Landsteiner and Chase 1935a); strains of bacteria,

isolated by Sickles and Shaw, which can degrade different pneumococcal carbohydrates (Sickles and Shaw 1934, 1935) and of which one, *Saccharobacterium ovale*, acts on group A substances as well (Landsteiner and Chase 1935b); and *Clostridium welchii* found by Schiff (1935) to attack group A substances. With regard to the decomposition of group A substances by other microorganisms, it may be mentioned that we found these preparations to be unaffected when incubated with a number of samples of decomposing vegetable matter; they were not altered by many common bacterial species, as Schiff (1935) has reported also, or by a chitinovorous bacterium[1] and several cellulose-decomposing organisms. In two cases, some degree of destruction of the A substance was detected after inoculation with rotting vegetables. Rather active decomposition was noted with a sample of aged, dry leaf mold and from this there was isolated the bacterium to be described.

Leaf mold was introduced into a medium comprising the A substance from commercial pepsin dissolved in the mineral base of Dubos and Avery (1931) (1 gram ammonium sulfate and 2 grams dipotassium phosphate per liter of water); after some days the culture medium had lost its specific property of inhibiting the hemolytic action (for sheep cells, in the presence of complement) of rabbit antisera to human group A erythrocytes. In isolating the organism, serial passages in 3 cc. portions of medium containing 0.02 per cent of the A preparation were employed in the beginning, but the persistence of extraneous bacteria in much greater numbers than the active organism necessitated plating on a medium containing A substance, mineral base and 1 per cent agar, the fishing of small areas of confluent growth (rather than single colonies) into the fluid A medium, and continued plating of such mixtures as were found after incubation to effect destruction of the specific substrate. After a few repetitions of this procedure, colonial fishings were practicable, and yielded a pure culture of the organism. As a precaution, eight consecutive platings from single colonies were made.

[1] A culture belonging to group I, supplied by the kindness of Dr. Anne G. Benton (1935).

For maintenance and study of the strain, two types of culture media were used. The first was a mineral medium containing 0.1 per cent of preparations rich in A substance, made from commercial pepsin and gastric mucin[2] (hereafter designated as "P" or "M" respectively), or slants of 0.5–1.0 per cent A substance and 1 per cent agar in mineral medium, employed usually at pH 7.0 to 7.2 (the optimum range is pH 6.0–6.5) because of moderate acid production during the growth of the bacteria.[3] After a preliminary study of the organism a second type of medium, termed "SP", was developed, based upon a utilizable carbohydrate and proteose peptone (Difco), which supports growth, probably chiefly because it has a relatively high content of group-specific A substance. The medium consists of 1 per cent Difco proteose peptone, 1 per cent sucrose, 0.1 per cent $(NH_4)_2SO_4$, 0.2 per cent K_2HPO_4, with 1 per cent agar as desired, in tap water, adjusted preferably to pH 6.5: the sucrose was added as a sterile solution to the autoclaved base. It should be mentioned that after cultivation for over a year on this "SP" medium the organism showed some decided alteration in cultural characters, as contrasted with a line maintained on medium "M".

When first isolated in pure culture, the bacterium was transplanted to a variety of common substrates, as well as synthetic media containing ammonium lactate or asparagin and tryptophane. There was no evidence of growth except in the cases of rabbit-blood beef-infusion Witte peptone agar, and litmus milk. On the former, a few small colonies slowly developed; with continued cultivation, the organism could be adapted to a scanty, unsatisfactory growth on beef infusion Witte peptone agar, again probably because of A substance in the peptone.

[2] The latter material, as used in the media, was prepared from crude gastric mucin (Wilson and Co., No. 1701-W) by heating a slightly acidified 2 per cent aqueous solution at 100°C., discarding material sedimenting upon centrifugation, and selecting the alcohol-insoluble fraction precipitating between ¾ and 2 volumes of alcohol, substances insoluble at the lower alcohol concentration first being removed by Berkefeld filtration; the material was reprecipitated once by addition of 2 volumes of alcohol to an aqueous solution.

[3] Whenever growth was evident in fluid media "M" or "P" (within 20 to 48 hours), degradation of the A substance could be confirmed serologically.

PURE CULTURE STUDY

General

For the determination of cultural characters, the organism was examined, when appropriate, on both "SP" and "M" media. For other substrates, 1 per cent proteose peptone in mineral medium was employed as a basal fluid, and growths on proteose peptone were used as seeding cultures. For nitrate reduction, 0.1 per cent potassium nitrate was added to this solution, while indol production by the organism was examined on the proteose peptone solution alone and with added "tryptone" or with 0.03 per cent tryptophane. Cellulose digestion was tested on fluid and solid media containing amorphous cellulose, prepared according to the method of McBeth (1916), in the peptone base or in medium "M". In making the biochemical tests, procedures recommended in the Manual of Methods for Pure Culture Study of Bacteria were followed.

Morphology

The best preparations were made from growths on fluid medium "M" at pH 5.5 to 6.5, incubated at temperatures between 25° and 32°C. for one to four days; the cells were then fairly uniform and stained clearly, the organism being a coccoid rod, occurring singly and in pairs, the somewhat oval forms measuring about 0.3–0.6μ by 0.5–0.7μ. On agar medium "SP" at pH 6.5, the individuals vary in size to a greater degree than noted above, averaging about 0.5 by 0.6μ, but large cells 0.9 by 1.2μ are also seen. On fluid medium "SP" at pH 6.5, the cell is not sharply outlined, and there is some variability in the intensity of the stain. Organisms grown in fluid media at reactions more alkaline than pH 6.5 stain increasingly poorly the higher the pH value, and rapid and complete autolysis occurs in but weakly alkaline fluids, e.g. upon treatment with dilute ammonium hydroxide.

Carbohydrate utilization

The organism did not grow on solutions of the test carbohydrates in the absence of proteose peptone. In media at pH 7.0 having 1 per cent carbohydrate and 1 per cent proteose peptone in the mineral base, a greater final amount of growth than

in the peptone control occurred in the presence of glucose, lactose, sucrose, galactose, mannose, xylose, maltose, inulin and dextrin, whereas mannitol, salicin and glycerol appeared not to be utilized.[4] The rate of development of the organism was markedly retarded over that in the peptone control by the presence of mannose, xylose, or salicin. There were only inconsiderable increases in acidity, about 0.2 pH unit, with the most vigorous growths; further studies with slants (1 per cent agar) made from sugar-peptone solutions and indicators revealed the development of acidity during growth on lactose (pH 6.6) and glucose (pH 6.8), but no or only insignificant changes in reaction with sucrose, galactose and maltose. No gas could be detected in Smith tubes (or in agar stabs) during active utilization of added carbohydrates; the growth was however limited to the surface of the open arm.

CULTURAL CHARACTERS

Coccoid rods, 0.3 to 0.6 by 0.4 to 0.9 microns, occurring singly and in pairs. Non-motile. Gram-negative. No spores demonstrated.

Gelatin "M" stab: no liquefaction.

Agar colonies: On beef-extract agar, no growth. On 0.75 per cent "M", 1 per cent agar: after two days at 32°, visible colonies 0.75 mm. in diameter, increasing in size to 5–7 mm. in 12 days. Circular, smooth, slightly raised, with an opaque raised dot set in a central depression. The internal structure appears coarsely granular. By transmitted light, bluish with thin light margins, by reflected light faintly yellow.[5] The consistency is butyrous to viscid. On this medium, only, daughter colonies often arise near the periphery of the parent colony after the fifth day. On "SP" 1 per cent agar: as above, except that the colonies are opaque and somewhat smaller. On blood agar (usual beef infusion peptone base): after 17 days at 32°, 0.75 mm. diameter, circular, raised, glistening, rather white.

[4] The utilization of the carbohydrates was confirmed by means of the Hanes' modification (1929) of the Hagedorn Jensen method for the microestimation of reducing sugars; media containing sucrose, inulin, dextrin, salicin, or lactose were first subjected to acid hydrolysis. The author wishes to thank Mr. Robert A. Harte for these determinations.

[5] When the agar concentration is higher, the colonies are smaller and quite transparent at first, but a decided brownish yellow color eventually develops.

Subsurface agar colonies: On 0.75 per cent "M", 1 per cent agar: distinctly brownish, often branched or lanceolate, opaque.

Agar stroke: On 1 per cent "M", 1 per cent agar: after 5 days at 32°–35°, moderate growth, glistening, contoured, flat, with margins rugose; translucent, slightly irridescent along margins; secondary colonial growths commonly observed along line of original streak; some irregular, cleared areas on old slant growths.

Infusion broth: no growth.

"M" or "SP" fluid media: growth at surface, settling to give an even turbidity.

Litmus milk: becomes slimy, reaction unchanged.

Lead acetate: slight browning?

No growth on potato.

Indol not produced.

Nitrates reduced.

No gas or pronounced acid production in carbohydrate media, but glucose, galactose, mannose, lactose, sucrose, maltose, xylose, inulin and dextrin are utilized. Very slight acid reaction detected with lactose and glucose.

Starch not attacked.

No characteristic odor.

Obligate aerobe; in agar shake cultures, growth does not occur more than 2 mm. below the surface.

Optimum temperature: about 32°; no growth at 40°C.

Thermal death, 2 day growth in "M" or "SP" fluids, after neutralization: 10 minutes at 51°C. not at 48°C.

Reaction of medium: pH limits of growth from 5.5 to 7.7, optimal range 6.0 to 6.5.

Cellulose agar not attacked.

Habitat: isolated from leaf mold.

CLASSIFICATION

The systematic position of a Gram-negative, non-motile, obligately aerobic, coccoid bacterium with the cultural peculiarities, as regards common sources of energy, of using certain peptones only, of growing either not at all or sparsely on common media, and—in the presence of peptone—of utilizing a number of sugars without frank production of acid or gas is difficult of decision.

A kinship with the two organisms in the genus *Saccharobacterium* erected by Sickles and Shaw (1934) in the family Mycobacteriaceae seems excluded on morphological grounds, also because of the failure of certain substances to inhibit growth when added to a favorable medium, namely the presence of 0.7 per cent sodium chloride, or 0.3 per cent beef extract, or peptone (a brand which was less satisfactory for supporting growth was tested for inhibition). The description given in Bergey's *Manual* of *Protaminobacter alboflavum* d would appear to permit a relationship, but a comparative study of bacteria of this type was not undertaken. It seems preferable, therefore, not to suggest a generic or species name for the A-splitting organism at the present time.

ACTION ON GROUP A SUBSTANCES

As already stated, the organism decomposes the group specific A substances from different sources, as human A saliva, horse saliva, and pig stomach (a material available in commercial pepsin and crude gastric mucin). The A preparations from pepsin and mucin have been used chiefly. During decomposition of the substances acid is formed (no gas is detectable by the common methods), and if the reaction is suitably readjusted to pH 6.5–7.0 at intervals during the period of incubation the organism will destroy the activity of 2 per cent solutions of the A preparations in the mineral base; this capacity for attacking the responsible serological structure is greater than that of *Saccharobacterium ovale* and exceeds by far the destructive activity of the *Myxococcus*. The action of the bacterium appears to lead to a thorough destruction, not for instance a mere splitting off of acetyl groups, since only traces of substances of polysaccharide nature were recovered following the action of the organism. No exoenzyme has been demonstrated. The bacterium did not attack the specific polysaccharides of *Pneumococcus* types I[6] or II[7], whereas *Pneumococcus* II polysaccharide is utilized by some other bacteria known to break down A substance, the *Myxococcus* of Morgan and *Saccharobacterium ovale* of Sickles and Shaw. In conclusion,

[6] The preparation (SSS, prepared by the older methods) was kindly furnished by Dr. Harry Sobotka.

[7] This material was supplied through the courtesy of Dr. W. F. Goebel.

the hope may be voiced that by means of such biological degradations of serologically active complex materials, information on the structure of these substances eventually may be yielded.

SUMMARY

The description is given of a bacterium having unusual characters and capable of destroying group A substances, that is, substances, presumably of polysaccharide nature, reacting with immune sera obtained by injection of human blood of group A. The organism does not grow on most common media, but can be cultivated in the presence of A substance.

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RELATIONSHIPS OF COLIFORM ORGANISMS

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The generally accepted practice in classifying members of the coliform group is to designate as *Escherichia* those strains which produce indol but do not form acetyl-methyl-carbinol and are incapable of utilizing citrate as the sole source of carbon. Parr (1936) has suggested that this group of reactions be termed "IMViC." Thus *Escherichia* is IMViC ++−−. Strains reacting in the inverse fashion (IMViC −−++) are designated as *Aerobacter*. A group with reactions resembling in part *Escherichia*, in part *Aerobacter* (IMViC −+−+) has been termed by some investigators "*Citrobacter*" while others prefer to include such organisms in the more general category of "intermediates."

With the standard IMViC differential tests there are 16 possible combinations of reactions. Moreover, if to these is added the ability to attack cellobiose (Koser, 1926) the number of possibilities is doubled, and if the production of acid or of acid and gas in the latter medium is taken into consideration there are theoretically 48 different types that could be obtained.

In an attempt to obtain as many of these types as possible and to study the frequency of their occurrence, some 5200 coliform cultures were isolated from human and bovine feces, dust of cow barns, and milk. Diluted fecal material was plated in eosin methylene-blue agar. The same procedure was used for milk except that in addition to eosin methylene-blue agar desoxycholate-lactose agar and "Bacto" crystal-violet red bile agar were also used. Eosin methylene-blue plates were exposed to the dust of cow barns and to material scraped from cow hides. All plates were incubated for 24 hours at 37°C. Typical coli-

form colonies were transferred from these plates to lactose broth. If acid and 20 per cent or more gas were produced, the broth was streaked on eosin methylene-blue agar and a well isolated colony selected for study. Indol, methyl red and Voges-Proskauer broths (Difco dehydrated), Simmons's citrate agar (Difco dehydrated), and cellobiose broths were inoculated with these cultures and incubated at 37°C. After 24 hours incubation the

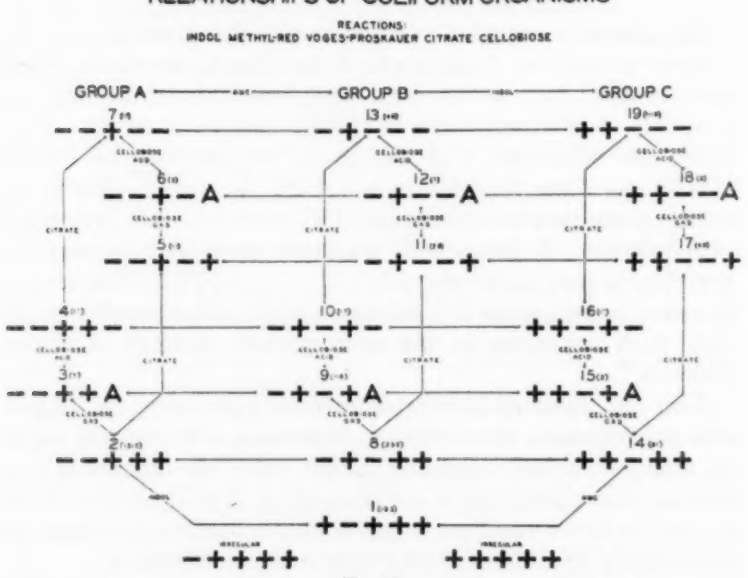

FIG. 1

indol broth was tested with Kovacs's reagent and the V-P broth with Levine's reagent. The M-R test was made with the standard reagent after 48 hours incubation. Reactions on the citrate agar and in the cellobiose broth were recorded daily for 5 days.

Of 4608 coliform cultures isolated and confirmed 3247 were grouped according to their reactions in the differential mediums. These cultures fall in orderly fashion into 3 groups of 6 types

each, one type intermediate between 2 of the 3 groups, and 2 irregular types or a total of only 21 out of the 48 possible combinations. Figure 1 correlates the different types according to their biochemical activities. The numbers in parentheses, except where noted, represent the number of cultures giving the reactions characteristic of the type on the first test in the differential mediums. When new types were encountered, a number of cultures were plated on eosin methylene-blue agar and tested in the differential mediums 3 successive times. If the cultures were constant in all their reactions, they were placed in stock. Such cultures were studied at intervals over a period of from 3 to 9 months after isolation for changes in their biochemical reactions.

IRREGULAR TYPES

Early in the work a wide variety of color reactions was obtained in the M-R and V-P tests. To observe these reactions more closely the M-R V-P medium was pipetted in 4 ml. amounts into tubes selected for size and color of glass. In the test 0.1 ml. of the methyl red solution was used. Rather than to attempt to determine the pH accurately with so large a number of cultures 5 degrees of reaction were used in recording the results. The tubes were shaken and recorded as + when red, O^+ when intermediate between red and orange, O when orange, O^- when intermediate between orange and yellow, and − when yellow. In the V-P test 4 ml. of the reagent were used. The tubes were shaken, the reactions noted after 4 hours, and finally recorded after 12 hours at 37°C. as: strong, moderate, weak, trace, or negative according to the intensity of color.

Approximately 9 per cent of all cultures studied were positive to some degree in both the M-R and V-P tests. A majority of these cultures gave a moderate to strong V-P test with an M-R test varying from O^+ to O^-. Others were strongly positive in the M-R test with a weak, trace, or questionable reaction in the V-P test. Only 10 cultures, all isolated from one sample of milk, were strongly positive in both tests. When the M-R V-P medium was inoculated with these 10 cultures and tested daily

for 2 weeks the M-R tests were + for from 5 to 7 days, then changed to O^+ or O^- but did not become really negative at any time. The V-P reaction under these conditions was consistently positive. When plated on eosin methylene-blue agar and incubated at 37°C. such cultures produced small, discrete, *Aerobacter*-like colonies. On subsequent incubation at room temperature for 24 to 48 hours the colonies developed to the large, raised, moist, confluent colonies characteristic in every respect of typical *Aerobacter*. Colonies from these plates or transplants from the parent culture transferred to the M-R V-P medium and incubated at room temperature were M-R negative and V-P positive in 24 to 48 hours. Growth in this medium was more luxuriant at room temperature than at 37°C.

Cultures giving a O^+ to O^- M-R reaction and a strong to moderate V-P reaction produced large and small colonies on eosin methylene-blue agar at 37°C. Large colonies gave a O^- to − M-R reaction at 37°C. and when replated produced both large and small colonies, the former predominating. Small colonies gave a + to O^+ M-R reaction and when replated produced both large and small colonies, the latter predominating. Both large and small colonies produced only large colonies at room temperature. Both large and small colonies when incubated at room temperature produced a negative M-R reaction over a 2 to 5 day period and a V-P reaction which might be strongly positive on the first day and weakly positive on the fifth day. Suitable experiments show that such cultures can utilize acetyl-methyl-carbinol and that the destruction is more rapid at room temperature. None of the 3247 cultures which produced both acid and gas in lactose, however, was negative in both the methyl-red and Voges-Proskauer tests.

Some cultures were isolated that produced a strongly positive M-R reaction and a weak or trace V-P test. These cultures produced typical intermediate or *Escherichia*-like colonies on eosin methylene-blue agar at 37°C. or room temperature, and the biochemical reactions were identical at both temperatures. Such cultures lost their ability to produce even a trace of acetyl-methyl-carbinol after from 3 to 9 serial platings or transplants. In these cultures a temperature effect does not seem to be in-

volved and a characteristic is lost. Attempts to build up this character by selection within such a culture failed.

A further characteristic of the irregular types—and also of type 1—is the low volume (20–50 per cent) of gas produced in fermentations. While the volume of gas produced by coliform organisms is admittedly a variable factor, nevertheless we were frequently able to predict that a culture with the colonial characteristics of an *Aerobacter* but with a low gas volume would have the biochemical reactions of an irregular or of a type 1.

Type 1

The colonial characteristics of type 1 are those of *Aerobacter* except that the tendency toward confluence is not marked. Over 90 per cent of these cultures produced a low gas volume. No motile form was found, although all other types with a significant number of cultures showed both motile and non-motile forms. (The fact that motility is not included as a characteristic in figure 1 does not imply that we attach no significance to motility within a type.)

To test the constancy of the biochemical reactions, a varying number of cultures of the different types, whose biochemical reactions had remained unchanged after 3 successive platings and tests in the differential mediums, were transplanted serially on agar and tested in the differential mediums after each transplant. After 3 or 4 transplants the number of cultures being studied was usually reduced. For example: 22 cultures of type 1 were transplanted from agar to agar 3 times and their biochemical reactions tested after each transplant. Since no changes in biochemical reactions occurred, the number of cultures under observation was reduced to 12 for the next 2 transplants and tests, with a subsequent reduction to 5 for the next course of 3 transplants and tests. Thus, 22 cultures of type 1 showed no change in their biochemical characteristics after from 3 to 8 transplants over a period of from 1 to 6 months after isolation.

Groups A, B, and C

It will be seen (fig. 1) that the 3 major groups are aligned on the basis of their reactions in the indol and M-R V-P mediums.

Within the individual groups the biochemically most active types are at the bottom of the grouping with characteristics lost in ascending order.

Most of the cultures in groups A and B were isolated from sources other than feces. Of the 1310 cultures of type 2 only 39 came from feces, while no cultures of types 3–7 were isolated from this source. Of the 471 cultures in group B, 29 came from feces and the remainder from milk, dust, and hides. All 28 cultures of type 11 were isolated from milk. In group C, types 14 and 17 were isolated from all sources except cow feces and type 19 from all sources. The number of cultures in types 15 and 18 are too small to be significant as to source.

Type 9 is the only type in any group in which the number of cultures isolated is comparable to the number of cultures of the basic type of the group. An analysis of our data shows the following fact which may or may not be significant. During the period from the first of May to the middle of June there were isolated from all sources 182 cultures of type 8 and 11 of type 9. From the middle of June to the middle of August 23 cultures of type 8 and 96 cultures of type 9 were isolated, and from the middle of August to the first of October 50 cultures of type 8 and 9 of type 9 were isolated. The total number of coliform cultures isolated in the first period was greater than in the second period, while the total number isolated in the second period was greater than in the third period.

There are certain relationships in colonial form among the groups and types which have been observed. Thus, in group A, cultures of type 2 were generally of the large, moist, confluent sort typical of *Aerobacter*. Other types (except type 7) resembled *Aerobacter* in general although the colonies appeared to be more discrete and somewhat smaller in size. Type 7 produced a discrete, bluish, raised colony 3 to 5 mm. in diameter with a slight metallic luster. Colonies of group B were for the most part indistinguishable from *Escherichia* colonies except for types 8 and 9, many of which produced small, somewhat rough, dry colonies with an intense metallic luster. Colonies of group C were typical *Escherichia*, except for type 14 which were larger

than ordinary and had a tendency toward confluence although they still retained a metallic luster.

The only type consistently producing a high gas volume (50–100 per cent) is type 2. All other types, even those in group A, produce less than 50 per cent gas. Indeed, type 7 never produced more than 20 per cent gas in 48 hours.

Study of the individual types during the course of successive transplants over a period of months indicated that certain strains were undergoing changes in their biochemical activities. These "shifts" in type are indicated in table 2. Certain salient features are immediately evident. Thus, in groups A and B, the shifts,

TABLE 1

Types of coliform organisms isolated from various sources

SOURCE	NUMBER OF CULTURES TESTED	TYPES																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Milk	1358	106	690	0	0	10	4	17	200	67	12	28	0	7	28	0	0	35	0	154
Human feces	278	3	39	0	0	0	0	0	5	5	0	0	0	2	4	2	0	3	0	215
Cow feces	383	0	0	0	0	0	0	0	0	0	0	0	0	17	0	0	0	0	0	366
Hides	528	38	258	4	0	1	0	0	23	4	0	0	3	5	5	0	0	3	2	182
Dust	700	46	323	3	0	0	1	0	27	40	5	0	4	17	24	0	0	8	0	202
Total	3247	193	1310	7	0	11	5	17	255	116	17	28	7	48	61	2	0	49	2	1119

with a single exception, involve the acquisition of biochemical activity. It is possibly significant that shifts are confined within the group with 2 exceptions, in which cultures of types 8 and 9 shifted to types 14 and 15 respectively, a shift from group B to group C. In this case, however, the shift from type 8 to type 14 is a shift in basic types and the cultures of type 8 involved were in a state of flux having just shifted from type 11 to type 8. Group C is peculiar in that it shows shifts to atypical forms (7 instances) more frequently than other groups. Shifts are not always in the direction of acquisition of characteristics as in groups A and B. There are 2 shifts, type 14 to 15 and type 18 to

19, which involve losses in ability to attack cellobiose. Changes in utilization of citrate do proceed in the direction of acquisition. A further observation on group C which was not sufficiently well

TABLE 2

Shifts in reactions of coliform cultures

	ORIGINAL TYPE	SHIFT TO TYPE	NUMBER OF CULTURES STUDIED	NUMBER OF CULTURES SHIFTING	NUMBER OF TRANSPLANTS TO PRODUCE SHIFT	CHANGE IN REACTION
	1		22	0		
A	2	1	25	2	3–5	+ Indol
	3	2	2	1	9	+ Gas cellobiose
	4		1	0		
	5	2	8	2	4	+ Citrate
	6		3	0		
	7	4	11	7	4–6	+ Acid cellobiose
	7	6	1	1*		+ Citrate
B	8		10	0		
	8	14	13†	2	4	+ Indol
	9		6	0		
	9	15	1‡	1*		+ Indol
	10	9	10	1	6	+ Acid cellobiose
	11	8	13	13	2–5	+ Citrate
	12	13	2	1	4	− Acid cellobiose
	13		5	0		
C	14	15	8	2	4	− Gas cellobiose
	15	Atypical	2	2	2–3	− Gas lactose
	16		1*	0		
	17	14	13	2	2–6	+ Citrate
	17	Atypical	13	5	2–6	− Gas lactose
	18	19	2	2	1	− Acid cellobiose
	19	16	19	3	2–4	+ Citrate

* Strain obtained by colony selection.
† Secondary shift following Type 11–Type 8 shift.
‡ Strain obtained from Type 10–Type 9 shift followed by colony selection.

defined to warrant inclusion in the table involves a tendency of 8 non-motile cultures of type 14 to acquire an ability to produce acetyl-methyl-carbinol to some degree in from 2 to 5 transplants. This tendency to shift to type 1 is all the more interesting when

it is noted that these 8 cultures are non-motile. It will be recalled that type 1 cultures are uniformly non-motile.

As is noted in table 2, not all of the shifts were obtained by the method of serial transplantation. Type 4 was never isolated from the specified sources. A culture of type 7 was plated on eosin methylene-blue agar and over 100 individual colonies fished and tested in the differential mediums. One variant was found that grew on citrate in five days. This strain was serologically identical with the parent strain. A similar result was reported by Minkewitsch and associates (1936). A culture of type 10 which acquired the ability to ferment cellobiose after 6 serial transplants (a shift to type 9) was plated on eosin methylene-blue agar in which the lactose had been replaced by cellobiose. Both black and white colonies were obtained. Black colonies produced acid in cellobiose in 24 hours and white colonies in 4 to 5 days. One of the colonies selected gave a weakly positive indol reaction after 2 transplants. On subsequent transplantation this culture increased in ability to produce indol, after which the characteristics remained constant.

Atypical coliform cultures

Approximately 600 cultures fished as typical *Aerobacter*, intermediate, or *Escherichia* colonies from dust, hides, and milk plates failed to confirm in lactose broth. Some of these cultures formed a bubble to 10 per cent gas in 4 to 6 days while others failed to produce any gas in 14 days. Such cultures when plated on eosin methylene-blue agar and incubated at 37°C. developed colonies which showed marked variation in size, ranging from those typical for a certain type to colonies barely visible. When left at room temperature, all the colonies on a single plate became comparable in size to those typical of the particular type.

Ninety-three of these cultures were selected for further study. They were replated, the differential mediums inoculated, incubated at 37°C. and at room temperature and tested after 36, 60, and 96 hours incubation. The reactions of representative cultures are shown in table 3.

Some cultures which produced no gas in lactose, were weakly

TABLE 3

Reactions of some atypical cultures

CULTURE NUMBER	TIME	37°C.						ROOM TEMPERATURE					
		Lactose	Indol	M-R	V-P	Citrate	Cellobiose	Lactose	Indol	M-R	V-P	Citrate	Cellobiose
	hours												
16 A	36	A	−	0^{+}	−	−	A	20	−	+	−	$+^{m}$	30
	60	A	−	0^{+}	−	−	B	20	−	+	−	$+^{s}$	30
	96	B	−	0^{+}	−	−	B	20	−	+	−	$+^{s}$	30
80 A	36	A	−	0^{-}	−	−	−	20	−	+	−	$+^{s}$	10
	60	A	−	0^{+}	−	−	A	20	−	+	−	$+^{s}$	20
	96	A	−	0^{+}	−	−	A	30	−	+	−	$+^{s}$	30
295 A	36	A	−	0^{-}	−	−	A	40	−	−	$+^{m}$	+	20
	60	A	−	0^{-}	−	−	A	60	−	−	$+^{s}$	$+^{s}$	30
	96	B	−	0^{-}	$+^{w}$	$+^{w}$	B	60	−	−	$+^{s}$	$+^{s}$	40
261 A	36	A	−	0^{+}	$+^{tr}$	−	−	30	−	−	$+^{s}$	+	30
	60	A	−	0^{-}	$+^{m}$	−	−	50	−	−	$+^{m}$	$+^{s}$	30
	96	A	−	0^{-}	$+^{w}$	−	A	80	−	−	$+^{m}$	$+^{s}$	50
366 A	36	A	−	0^{+}	−	−	−	20	−	+	−	$+^{s}$	30
	60	A	−	0^{+}	−	−	A	20	−	+	−	$+^{s}$	30
	96	A	−	0^{+}	−	−	A	20	−	+	−	$+^{s}$	30
457 A	36	A	+	0^{+}	−	−	B	20	+	0^{-}	$+^{m}$	+	20
	60	A	+	0	−	−	B	30	+	0^{-}	$+^{s}$	+	30
	96	A	+	0	−	$+^{w}$	10	30	+	−	$+^{m}$	$+^{s}$	30
499 A	36	−	−	0^{+}	−	−	−	20	−	0^{+}	−	+	20
	60	A	−	0^{-}	−	−	−	20	−	+	−	$+^{s}$	30
	96	A	−	0^{+}	−	−	A	20	−	+	−	$+^{s}$	30
628 A	36	B	+	+	$+^{w}$	−	10	20	+	−	$+^{m}$	$+^{s}$	40
	60	20	+	0^{+}	$+^{m}$	$+^{w}$	40	20	+	−	$+^{m}$	$+^{s}$	40
	96	20	+	0^{-}	$+^{m}$	$+^{m}$	40	20	+	−	$+^{w}$	$+^{s}$	40

$+^{tr}$ = trace.
$+^{w}$ = weak.
$+^{m}$ = moderate.
$+^{s}$ = strong.
A = Acid in carbohydrates.
B = Bubble of gas.
Numbers in carbohydrate columns indicate per cent of gas formed.

positive in the V-P test, and failed to grow on citrate at 37°C., were typical type 2 at room temperature. Others were V-P positive and citrate negative at 37°C. and positive in both reactions at room temperature. One culture (261A) which produced only acid in cellobiose at 37°C. and this only after 96 hours, produced acid and 50 per cent gas in this medium after a similar period at room temperature. The production of indol appears to be the same at both temperatures.

Of the 93 atypical cultures studied 53 produced acid and gas at room temperature. Thirty of these possessed the biochemical characteristics of type 2, 21 of type 8, and 2 of type 1. The remaining 40 cultures failed to produce gas at room temperature. Some of these were indol and M-R negative, strongly positive in the V-P and citrate mediums, produced acid in cellobiose and subsequently developed a red pigment (*Serratia*). Six cultures, all from milk, had the reactions of type 15 (++−+A) and one the reactions of type 19 (++−−−). No atypical culture producing indol which produced gas only at room temperature was obtained on primary isolation.

Several cultures isolated as atypical types 2 and 8 were found to produce gas in lactose at 37°C. after being in stock without transplanting for from 2 to 3 months. Typical type 2 cultures occasionally lost their ability to produce gas at 37°C. so that shifts from atypical to typical and from typical to atypical occur not infrequently.

Plant "coliform" cultures

In view of the source of all our atypical cultures—milk, dust, and hides—their biochemical reactions and particularly the inability of some cultures to produce gas from lactose at 37°C., a possible relationship to the organisms in the tribe Erwinieae is obvious. Accordingly eosin methylene-blue plates were streaked with decayed portions of a number of fruits and vegetables. After 48 hours at room temperature 200 coliform colonies were transferred to lactose broth and agar slants. Forty-two cultures, because of differences in colonial characteristics and reactions in lactose broth, were selected for further study.

The cultures were replated, transferred to lactose broth, and incubated at 37°C. and at room temperature. Thirteen cultures produced acid and gas at both temperatures, 24 produced gas at room temperature only, and 5 produced acid but no gas at either temperature. The reactions of these cultures in the differential mediums were determined at room temperature. Of the cultures producing gas, 6 were type 1, 21 type 2, 6 type 8, 1 type 9, and 3 type 14. Three cultures not producing gas at either temperature were $++--$A and 2 were $-+--$A.

It was hoped that the plant coliform cultures would, in most respects, resemble or be identical with the atypical cultures. There were certain differences apparent when the plant cultures were first isolated, but these became less marked on further cultivation under laboratory conditions. While a small percentage of typical and atypical cultures produced strong odors, the majority of the plant cultures did so when first isolated. The odor disappeared rapidly when the cultures were maintained under laboratory conditions. In lactose broth the plant cultures grew much more luxuriantly with a tendency toward ropiness which disappeared on subculturing. In the majority of atypical cultures the V-P reaction was moderate to strong. The plant cultures on primary isolation gave a trace to weak reaction if they were positive at all but the intensity of the reactions increased to moderate to strong over a period of 3 months. One hundred per cent of the plant cultures and all of the atypical cultures but one produced either acid or acid and gas from cellobiose. It is possible that the differences noted between the plant cultures and the atypicals may be due to differences in natural environment since the strains tend to become more alike when cultivated under the same conditions.

Serological investigations now in progress seem to show an antigenic relationship between the plant, atypical, and typical coliform organisms.

DISCUSSION

We realize that certain terms and procedures used in this paper require explanation. There may be some objection to selecting

definite time limits (24 hours for indol and V-P tests, 48 hours for the M-R test, and 5 days for the reaction on citrate and cellobiose) in recording the reactions. We are quite in agreement with Gray (1932), Ruchhoft and associates (1931), and others in their observations that the time element is extremely variable. This is especially true in the case of the M-R reaction, where, had we allowed 5 days incubation, few irregulars would have been encountered. Cultures on citrate might in some cases develop a positive reaction in 2 or 3 weeks whereas they were completely negative in 5 days. The same is true of the reaction in cellobiose. Nevertheless these cultures were recorded as negative. The setting of a definite limit is necessary in comparing the reactions of over 3000 cultures where more than 8000 sets of differential tests are involved, and the limits we have set have proved very satisfactory for the great majority of cultures.

In describing our experimental results we have had occasion to refer to "shifts" in type of certain of our cultures. Ruchhoft and associates (1931) stress the necessity of purifying cultures before assigning them a definite position in classification. We are quite in agreement with this point of view, and all of our cultures were treated in a manner wholly analogous to that of these workers in "purifying" their cultures. All strains were replated and tested 3 times for constancy of reaction. We prefer to use the term "stabilization" for this treatment of the cultures since "purification" implies contamination by a foreign species and we have worked with strains as pure as is possible to obtain them short of single cell isolation. "Stabilization" implies a reasonable constancy of reaction without excluding the possibility of variation under suitable conditions.

While all our cultures were uniform in colony type and constant in reaction under the conditions previously noted, there were two situations which were encountered in treating the cultures in which constancy of cultural characteristics was apparently lacking. When eosin methylene-blue plates were streaked from the original lactose broth we found occasionally two or more colony types on the plate. Such findings were undoubtedly due to mixed cultures in the sense in which Ruchhoft

refers to them. The individual types were, however, constant in their reactions after stabilization. On the other hand we have described certain atypical cultures which produced apparent mixtures of colony types at 37°C. while producing uniform types at room temperature. In this instance the effect is obviously a temperature characteristic of the strain and does not, we believe, imply a contamination of the culture.

In the work reported here it seems hardly likely that the orderly manner in which shifts occur could be entirely fortuitous. Were the results due to contaminations we would anticipate that the shifts would in most cases be to totally dissimilar types rather than to those closely related to the parent strain. Ruchhoft in one instance describes the isolation of three colonies from one of his purified strains which varied markedly in the manner in which they produced acetyl-methyl-carbinol. Some of our observations, especially on the tendency of type 14 to shift to type 1, are similar in import to these findings. With the exceptions noted our observations on shifts in reactions have been made on whole cultures in serial transplants. Thus we have been observing the activity of the entire bacterial population and the changes appear to be gradual. The problem is being reinvestigated at present by the method of colony selection to determine if possible whether the changes in type are gradual or appear suddenly in individual colonies. Another point that we hope will be clarified by this method is the nature of our so-called "irregular" strains, especially those which are strongly positive in both the M-R and V-P tests. On theoretical grounds we would anticipate perfect, or nearly perfect, negative correlation between these two tests. It may be, as Ruchhoft suggests, that cultures of this type are mixtures even though they have been stabilized and seem to be constant in their reactions. Our strains have been constant in their reactions over a series of 3 to 12 transplants and the "shifted" strains have remained stable for from 3 to 5 transplants.

It has been suggested by a number of investigators that the irregularities in the M-R and V-P reactions are due to lack of sufficient incubation time before performing the tests. While

this is probably true, there can be little doubt that there is marked variation in the rate of reaction within individual strains. Thus Koser (1924) describes 8 strains which were M-R positive, V-P negative immediately after isolation which gradually changed until after a month or more they were M-R negative, V-P positive. This, of course, was under standardized conditions of testing. A similar condition has been observed in some of our atypicals and, as previously noted, 8 strains of type 14 showed a tendency to shift in this manner.

These irregularities in the M-R V-P reactions have been most noticeable in the so-called "atypical" strains, which, it will be recalled, are strains which either fail to produce gas from lactose or produce gas at room temperature only. Parr (1934) has noted that cultures are frequently isolated from fresh feces which are lactose-deficient and comments that such strains can usually be "trained" back to normal. We have noticed a similar acquisition of the ability to produce gas from lactose in many cultures, notably the plant "coliform" cultures. The fact that the acquisition of a positive V-P reaction appears to correlate with the development of the ability to produce gas from lactose leads us to suggest a possible mechanism for the change.

A probable series of reactions resulting in the formation of acetyl-methyl-carbinol involves the decarboxylation of pyruvic acid with subsequent condensation of the resulting acetaldehyde. Since, in fermentations, pyruvic acid is thought to result from the dehydrogenation of methyl-glyoxal hydrate, the formation of the precursors of acetyl-methyl-carbinol is a result of the action of the gas-producing enzymes. Sizer (1938) has suggested that the situation may be analogous to the course of certain enzyme-catalyzed reactions in which the end-point may be reached by more than one pathway. The energies of activation of the enzymes involved may so vary with temperature that one series of reactions will predominate over one temperature range while a second series will come to dominance as the temperature changes. It may be that the failure of certain strains to produce gas from lactose at incubator temperatures may be due to the dominance of enzymes leading to acid end products. Such

a concept, or course, involves the assumption that those strains which acquire the gas-producing ability over a series of transplants eventually lose the ability to produce enzymes of this type. Koser (1924) comments: "It seems probable . . . that the secondary or alkaline fermentation was 'speeded up.' The production of acetyl-methyl-carbinol was never apparent until this reversion had taken place."

As to the validity of the types presented in figure 1 there can be little doubt. Skinner and Brudnoy (1932) although they made no distinction between the production of acid and of acid and gas in cellobiose, have described types which correspond to all save types 3–7, and 11 and 12. Oeser (1937), who records the production of gas in cellobiose, has isolated all types save types 4–7 and types 15 and 18. Both authors report strains which we would group with our irregulars.

At present we are reluctant to stress too strongly the "evolutionary" aspects suggested by the grouping in figure 1 and by the observed shifts in type. A number of investigators have commented on evolutionary tendencies in the coliform group and their possible origin from more primitive forms. Winslow and associates (1919) give what might be termed a "metabolic gradient" of the colon-typhoid group based on fermentative capacities although the authors state: "It cannot, however, be maintained that this progression necessarily or even probably represents the exact line of evolutionary development." The close similarity in the metabolic activities of the genus *Serratia* and members of the genus *Aerobacter* is well known, and, as Pederson and Breed (1928) point out, non-pigmented strains of *Serratia* may easily be mistaken for *Aerobacter*. Frobisher (1937) refers to the *Serratia* group as "infra-aerogenes."

Certain of our recorded observations appear to suggest such possibilities. Considering the basic type in group A (type 2): cultures have been obtained which vary in gas production all the way from complete inability to produce gas at any temperature up through types which produce 100 per cent of gas at incubator temperature. The basic type of group B (type 8) shows similar relationships except that the maximum volume is less than 50 per cent.

The numerical relationships between the types in the different groups suggest that in their natural habitat the organisms may "evolve" into types 2, 8, and 19. The nature of the shifts observed tends to confirm this concept, although we are at a loss to explain the relative scarcity of type 1. It may be that such "evolutionary" activity, if it exists, proceeds from two directions with a tendency to stabilize at the basic types. Until more data are available we feel justified only in calling attention to the fact that changes in type seem to follow closely the scheme laid down in figure 1.

The results reported in this paper do, we believe, contribute in some degree to the question of classifying the members of the coliform group. Suggestions for the classification of this group range all the way from the creation of a separate genus, "*Citrobacter*," (Werkman and Gillen, 1932) to the elimination of all save the species *Escherichia communis* and *Aerobacter aerogenes*. In the middle ground Tittsler and Sandholzer (1935) deem it possible to allocate the intermediate types to the genus *Escherichia* with the distinction between *Escherichia* and *Aerobacter* based on the production of acetyl-methyl-carbinol. Carpenter and Fulton (1937) also stress the inadvisability of creating a separate *Citrobacter* genus. On the other hand the frequency with which intermediate types are encountered makes us feel that it would be unwise to regress too far in the direction of simplification. The importance of this group of organisms in the determination of the sanitary quality of water, milk, shellfish, and other food-stuffs makes it desirable that we retain a distinction between the various groups.

By grouping the members of the coliform section as indicated in figure 1 it is possible to place the great majority of types encountered with a minimum of effort and expense in one of three fundamental groups. This can be accomplished by the use of but two reactions, indol and Voges-Proskauer (or methyl-red). For more careful classification, citrate and cellobiose may be used, but in the majority of instances this would prove unnecessary and even confusing.

The subdivision of the coliform section into three major groups seemed most suitable in view of the numerical relationships of

the various types and the direction of shifts in type. Statistical analysis of the data confirms this concept and serves to place it on a less empirical basis. Winslow and Rogers (1906) and Levine (1918) have applied correlation coefficients to the problem of classification. While distinction between species should be based on those reactions showing the least correlation, major subdivisions of a large group of organisms should be characterized by reactions as highly correlated as possible. The statistical treatment of our data indicates that the grouping given in figure 1 most nearly accomplishes this purpose. Tetrachloric coefficients of correlation (table 4) were computed for all the reaction pairs used in differentiating the members of the coliform group. The distinction between types within a given group is based on the reactions in citrate and cellobiose, which are the least

TABLE 4

Coefficients of correlation of reaction pairs in differential mediums

	INDOL	M-R	V-P	CITRATE	CELLOBIOSE
Indol		0.90	−0.90	−0.97	−0.98
M-R	0.90		−1.00	−0.98	−0.99
V-P	−0.90	−1.00		0.98	0.99
Citrate	−0.97	−0.98	0.98		1.00
Cellobiose	−0.98	−0.99	0.99	1.00	

correlated of the reaction pairs. Separation into three major groups on the basis of indol and V-P (or M-R) reactions serves the same end in that these reactions are thus perfectly correlated within the group whereas they would not be were the distinction made on the basis of V-P and citrate.

We would suggest, then, that the members of the coliform group of organisms might be divided into three sections on the basis of their reactions in indol and Voges-Proskauer mediums. The sections might be termed *Aerobacter*, Intermediate, and *Escherichia*.

SUMMARY

1. Organisms of the coliform group (3247 strains) fall into 3 major groups of 6 closely related types each.

2. "Irregular" and "atypical" strains encountered may represent primitive forms of the coliform group.

3. It is suggested that the coliform group of organisms be divided into 3 sections: *Aerobacter*, Intermediate, and *Escherichia* on the basis of their indol and Voges-Proskauer reactions.

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COLIFORM ORGANISMS IN CERTIFIED MILK

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While investigators agree that coliform organisms in milk are of little significance with respect to conditions of production, a number have suggested that colon counts on high quality milks may be of some value under certain conditions. Ayers and Clemmer (1918) and Sherman and Wing (1933) suggest that the presence of coliform organisms is significant only if the total bacterial count is less than 10,000, the milk is relatively fresh, and the holding temperature has been below 50°F. Certified milk is required to meet these conditions. The American Association of Medical Milk Commissions has approved a standard of less than 10 coliform organisms per milliliter, and has recommended the use of desoxycholate agar mediums (Leifson, 1935) for plating. Two types of desoxycholate agar mediums are available commercially. These differ primarily in their concentration of sodium desoxycholate. The higher concentration (0.1 per cent) is recommended for certified milk or when 1 ml. of whole milk is to be plated, while the medium with the lower concentration (0.05 per cent), so-called "desoxycholate-lactose agar" is to be used when diluted milk is plated.

To obtain a comparison of the two desoxycholate mediums 80 samples of certified milk were plated in both. In addition 40 samples of producers' raw, a relatively low quality milk, were carried through the same procedure. Sixty of the certified and all of the producers' raw milks were also plated in eosin-methylene blue agar. The results of the comparative tests are shown in table 1. For each kind of milk the average counts on desoxycholate agar were only about 62 per cent of those on desoxycholate-lactose agar, even though the certified milks were plated

undiluted. Coliform counts on eosin methylene-blue agar were essentially the same as on desoxycholate-lactose agar. These comparative counts show that the medium containing the lower concentration of sodium desoxycholate is more satisfactory for the enumeration of coliform bacteria even in low count milks. The greater inhibition of non-coliform organisms by desoxycholate-lactose agar renders it superior to eosin methylene-blue, especially in routine examination of high count milk.

Weekly or semi-weekly samples of certified and producers' raw milks were plated, usually in desoxycholate-lactose agar. If coliform colonies developed they were carefully examined, representative numbers of the different kinds transferred to lactose broth, and the reactions noted after 24 hours. Such

TABLE 1

Comparison of plating mediums for coliform count

MILK	SAMPLES TESTED	COLIFORM PRESENT	AVERAGE COLIFORM COUNT ON		
			Desoxycholate agar	Desoxycholate lactose agar	Eosin methylene-blue agar
Certified	80	43	49	78	75*
Producer's raw	40	35	125,000	204,000	215,000

* Only 60 samples were plated on eosin methylene-blue agar.

confirmatory tests were applied to 2252 coliform colonies from certified and producers' raw milks. Acid and gas were produced by 81.1 per cent of the cultures and acid only by 18.9 per cent. ("Atypical" cultures, producing only acid in lactose, will be discussed in a subsequent paper.) Of the 1826 cultures producing acid and gas, 1106 were plated on eosin methylene-blue agar.[1] Indol, methyl-red and Voges-Proskauer broths, Simmons's citrate agar, and cellobiose broth were inoculated from well-isolated colonies. Indol and Voges-Proskauer tests were made with Kovacs's (1928) reagent and Levine's (1934) modi-

[1] Eosin methylene-blue agar: 12.5 grams proteose peptone, 200 mgm. eosin, 50 mgm. methylene blue, 10 grams lactose, 10 grams Bacto agar, 1000 ml. distilled water. On this modification of eosin methylene-blue coliform colonies can be differentiated easily from non-coliform colonies in from 12 to 15 hours.

fication of O'Meara's reagent, respectively, after 24 hours incubation at 37°C. Methyl-red tests were made after 48 hours incubation. Growth on citrate agar and the reaction in cellobiose broth were observed daily for 5 days.

During 6 months (April to September) 233 samples of certified milk from 5 farms were examined. The incidence of samples with coliform organisms is given in table 2. Cultures were recorded as *Escherichia*, intermediate, or *Aerobacter* according to the accepted reactions of the groups in the differential mediums.

TABLE 2

Incidence and sectional differentiation of coliform organisms from milk

MILK	SAMPLES EXAMINED	COLIFORM PRESENT	MORE THAN 10 COLI PER ML.	AVERAGE COLI PER ML. WHEN PRESENT	NUMBER SAMPLES FROM WHICH COLI WERE ISOLATED	CULTURES STUDIED	ESCHERICHIA	INTERMEDIATE	AEROBACTER
		per cent	*per cent*				*per cent*	*per cent*	*per cent*
Certified farm B	30	63	3	4	15	103	13.5	10.8	75.7
Certified farm C	60	98	78	97	23	229	0.0	47.2	52.8
Certified farm F	63	46	10	27	14	94	9.6	10.6	79.8
Certified farm W	31	48	0	1	12	47	27.7	8.5	63.8
Certified farm Fe	49	71	37	142	24	148	18.9	45.9	35.2
Total	233				88	621	10.3*	32.4*	57.3*
Producer's raw	71	80	76	147,000	52	485	9.1	16.7	74.2

* Per cent of 621 cultures studied.

Three certified milk farms were selected for investigation as to possible sources of the coliform organisms. Direct isolation on eosin methylene-blue plates was used. Sterile plates were exposed to the dust of the barns and dairies and to material scraped from the sides, bellies, and udders of cows. Samples of fresh cow feces were collected and dilutions plated in eosin methylene-blue agar. Coliform colonies from the various sources were transferred to lactose broth, confirming cultures plated on eosin methylene-blue, and selected colonies studied as

previously described. This procedure was repeated 3 times at each farm.

From the feces of 30 cows from the 3 herds, 383 coliform cultures were studied. One hundred per cent of these were *Escherichia* despite the fact that every colony with any *Aerobacter* characteristics was fished. It is obvious that only the *Escherichia* present in these milks could be due to the bacteria from cow feces. That *Escherichia* from this source should infect milk while the other types of coliform bacteria in the same milk come from entirely different sources seems quite improbable.

TABLE 3

Sectional grouping of coliform organisms from milk, stable dust, and cow hides

FARM	MATERIAL	COLONIES	ESCHERICHIA	INTERMEDIATE	AEROBACTER
			per cent	*per cent*	*per cent*
C	Milk	229	0.0	47.2	52.8
	Dust	320	18.5	12.8	68.7
	Hides	275	20.4	10.6	69.0
F	Milk	94	9.6	10.6	79.8
	Dust	189	81.5	5.8	12.7
	Hides	54	92.6	0.0	7.4
Fe	Milk	148	18.9	45.9	35.2
	Dust	278	14.7	27.7	57.6
	Hides	192	45.8	3.6	50.6

Farm "C" is particularly interesting in this respect since no member of the *Escherichia* section was isolated from its milk.

Table 3 shows the percentages of the 3 sections of coliform organisms found in the milk as compared with dust and hides. The results as given include examinations of the milks over 6 months, while the specimens from dust and hides were collected during 2 to 4 months. The diametrical opposition of the flora of the milk from farm "F" to that of the dust and hides is particularly striking, as is the fact that of the large number of cultures isolated from the milk of farm "C" none was of the *Escherichia* section. The differences in the case of farm "Fe" are less striking, but inasmuch as hand milking is practiced the

chances of air contamination are greater. Considerable variation in the percentage distribution at the different farms was observed, but in no case was the distribution similar to that observed in the milks.

DISCUSSION

Numerous mediums, both liquid and solid, have been recommended for the isolation and enumeration of coliform bacteria in milk. The merits of a large number of these have been studied and discussed extensively by Bartram and Black (1936) and Yale (1937a). Granted a suitable medium the major objection to the use of liquid mediums is the large number of tubes which must be used if the determination is to be quantitative. On the other hand, both these authors have found that a considerable number of the colonies developing on solid mediums fail to confirm in lactose broth. In the present study 18.9 per cent of such strains were encountered. Yale (1937a) has called attention to the difficulty of classifying these organisms and assigns his strains variously to the genera *Achromobacter*, *Flavobacterium*, *Serratia*, and *Alcaligenes*. Oeser (1937) has noted the close relationship of many of these organisms to the genera *Erwinia*, *Phytomonas*, and *Klebsiella*. A subsequent paper will discuss the reactions of these "atypicals" and their biochemical and serological relationship to the coliform group.

There is extensive evidence that the coliform count in raw milk in general is of little sanitary significance. (Ayers and Clemmer, 1918; Bartram and Black, 1937b; Kline, 1935; Sherman and Wing, 1933; Yale et al., 1935, 1937b.) In pasteurized milk the presence of the group has an entirely different meaning. (Chilson et al., 1936, Yale, 1937a, b.) In certified milk, however, the conditions are such as led Ayers and Clemmer (1918) and Sherman and Wing (1933) to suggest that the coliform count may serve as a supplementary index of sanitary quality. If, as Bartram and Black (1937) suggest, the tendency to regard all coliform organisms as of possible fecal origin is valid, then the presence of any members of the group would indicate a highly undesirable condition. Parr (1936, 1937) has noted a "succes-

sion of forms" in stored feces where the *Escherichia* types found in fresh samples soon disappeared and were replaced by intermediates and members of the *Aerobacter* group. Moreover many of the *Aerobacter* types were "lactose degraded" and might correspond to the "atypical" strains which we have encountered. On the other hand, Tonney and Noble (1931) found no significant change in relative numbers of *Escherichia* and *Aerobacter* as they occur in fecal material, nor was there a material difference in the survival time of the types.

Kline (1935), on the basis of percentage distribution of coliform types in milk of different grades, feces, hay and grain, and pasture soil, concluded that reliance should be placed on the identification of *Escherichia* if an index of fecal pollution were desired. On a similar basis (table 3) we would incline to the same opinion. The milk flora by no means showed any correspondence to bovine fecal contamination since no members of the *Aerobacter* or intermediate sections were encountered in cows' feces, and it must be admitted that the findings in dust and hides do not warrant the assumption that they are the sole source of contamination. In view of the high incidence of members of the *Aerobacter* section in these milks the findings of Ayers and Clemmer (1918) suggest the probability of utensil contamination as the source of the coliform organisms encountered.

Too much stress should not be placed on the occurrence of members of the coliform group as a whole in certified milk. The extensive observations of other investigators quoted herein and our own findings render the presence of members of the *Aerobacter* and intermediate sections insignificant as an index of fecal contamination, yet a rigid insistence on adherence to the standards set for the coliform count would result in the condemnation of such milks as that of farm "C" which had an average coliform count of 97 over a period of 6 months (table 2) with no member of the *Escherichia* section encountered.

SUMMARY

Classification of coliform organisms isolated from certified milk showed considerable variation in proportions of *Aerobacter*,

intermediate, and *Escherichia* types with a marked predominance of *Aerobacter* and intermediate forms. Since the three groups differ in their sanitary significance the total coliform count seems to be of doubtful value.

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A CINEMATOGRAPHIC ANALYSIS OF THE MOTION OF COLONIES OF B. ALVEI

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At the 1937 meetings of the Society of American Bacteriologists, Smith and Clark reported (1938) on the motility of entire colonies of *Bacillus alvei* and other organisms. Being interested at the time in lapse-time motion picture studies of bacterial colonies, we obtained a culture through the agency of the American Type Culture Collection. The marking on this culture leads us to assume that it came originally from Smith.

The strain was found to be motile as described. Preparation for cinematographic study comprised seeding one point on the margin of a freshly poured plate of 2 per cent Proteose-Peptone, beef infusion agar (1.5 per cent agar) of pH 7.4. The exact amount of surface moisture was found to be highly critical, and several plates of varying degrees of dryness were always set up. These were incubated briefly until the plate showing rapid motion of discrete colonies across the fresh medium could be selected with the aid of a hand lens. This plate was then placed in the incubator (30°C.) of the photographic equipment and a record made of about two days' growth at 15 frames per hour. This resulted in a 3800 times magnification of speed when the film was projected at the normal rate. On one hundred feet of film we have recorded the action on 10 plates.

The development of a typical culture appears to fall into five general periods:

1. The first growth consists of minute colonies which move across the field at high speed in any direction. The path is rarely linear for any distance. Rather, it is usually a series of wide circling movements. The number of such active colonies increases rapidly until the second phase sets in.

2. The colonies now come to rest, not gradually, but very abruptly. Frequently the small stable colony so formed will have a residual motion of rotation. The abruptness of the halt eliminates any question of "running down," and I think it is to be explained by an occurrence which is frequently seen under the microscope, namely, a turning back of the head of the advancing colony so that it forms a sharp left hand loop which quickly progresses to form a spiral and then, as the spiral tightens, a compact colony which still possesses the rotary motion to which its linear motion has been transformed.

3. When nearly all the colonies have entered upon the second phase, the third begins. This is one of comparative rest with continued growth in size.

4. The fourth phase embraces a second active period in which colonies apparently at rest take on a slow rotary motion which accelerates and becomes more extensive as time goes on. This is photographically the most striking stage. As the rate increases, spiral arms are extended from many colonies so that they resemble spiral nebulae. Fragments are frequently detached to lead an independent existence and may depart entirely or continue to circle the parent as a satellite. Some colonies will detach a complete ring of growth which continues to rotate about the center at some distance from it. These changes resemble those caused by centrifugal force, but this is, of course, illusory.

5. In the last stage the culture "freezes" into a final stability. In contrast to the abrupt halt in the initial phase, this stoppage is gradual. The spirals and rings rotate more and more slowly until finally all motion is lost. This seems in general to be the result of overcrowding but may be in part due to loss of surface moisture.

This last point is borne out by an accident which took place in one series. During the run the temperature in the incubator fell for a short time with a resultant condensation of moisture on the glass cover of the petri dish. In viewing the film it was noted that, simultaneously the culture, which was in the early 4th stage, ceased its rotations. When the damage was repaired, with consequent rise in temperature and redistillation of the

condensed water from the glass back to the surface of the now cooler agar, the motion was abruptly resumed. Since this motion does not appear to be impaired by room temperature as opposed to 30 degrees Centigrade, moisture would seem to be the controlling factor in this instance.

The timed nature of the lapse-time picture permits ready measurement and comparison of velocities on numbers of colonies. The following average values are typical:

Rotations (phase 4):	
Colonies 1–1.25 mm. in diameter	1.4 r.p.h.
Colonies 1.25–1.75 mm. in diameter	0.8 r.p.h.
Colonies 1.75–2.25 mm. in diameter	0.5 r.p.h.
Linear motions (phase 1):	
Colonies 0.2–0.5 mm. in diameter	14 mm./hr.

It may be interesting to compare the last value with stated velocities for single motile cells of other species.

Eberthella typhi	65 mm./hr.
Bacillus megatherium	27 mm./hr.
Bacillus alvei (colonies)	14 mm./hr.

When we consider the bulk of the *B. alvei* colony, as compared to that of a single cell, the value 14 appears to be quite respectable.

Another interesting observation is that, of the probably 200–300 rotating colonies shown in the film, only two have been detected whose motion is clockwise. The remainder are uniformly counterclockwise. This brings up again the controversy which has been raised at numerous times in the past over the reason for such directed rotation in the biological world.

To return finally to the paper by Smith and Clark, I must call attention to two points: first, the statement that (as distinguished from the motion of *Bacillus circulans*) "in the *B. alvei* type the colony moves forward like a bullet and may take any direction." It would appear evident from the motion picture studies that Smith's statement is true only for phase 1 and that in the later stages the most characteristic feature is a rapid and extensive rotation with no motion of translation. It is only fair to state that we did not have more than a vague suspicion of the existence of this rotary motion until the pictures were available. Second,

while not in a position to suggest the true mechanism, I must question the conclusion that "the motility of the colonies is not a characteristic of any one species, but that it is the result of a strong motility of the individual cells combined with certain physical conditions of the medium." I doubt if this can be the entire answer, for, while we may grant that the motive power is derived from the motility of the single cell and that the condition of the surface is a highly critical factor, we still leave unexplained the unified action of the cells so that the colony moves as a whole rather than spreading in all directions as in the case with other highly motile strains. It would seem necessary to postulate some mechanism by means of which the individualists of the cell population are induced to submit to a regimentation.

It is my impression that colonial motility in *B. alvei* has been described only in Smith's report. It is difficult to understand why such an outstanding characteristic should have been overlooked. In an article by Muto (1904) there is described an organism with strikingly similar properties. The motions of this strain (*Bacillus helixoides*) are apparently identical with those described here for *B. alvei*. The question is then raised as to whether the strain in my possession is *B. alvei* or *B. helixoides* or whether, in last analysis, these two species, which according to reported descriptions are distinctly different in morphology and staining, are one and the same. The strain in my possession would, on cursory examination, answer best to Muto's description.

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COMPARATIVE STUDIES ON THE PURIFICATION OF TETANUS AND DIPHTHERIA TOXINS

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Diphtheria toxin has been identified as a typical heat-coagulable protein (Eaton 1936 a, 1937; Pappenheimer 1937). If other toxins have chemical properties similar to diphtheria toxin, they might be isolated by similar methods.

Using a gelatin hydrolysate medium, Pappenheimer and Johnson (1936, 1937) have produced diphtheria toxin which may be obtained in a relatively pure state by simple fractionation with ammonium sulphate. However, strong tetanus toxin has not been produced on a simplified medium similar to that used by Pappenheimer and Johnson for diphtheria toxin. Consequently, attempts to isolate tetanus toxin must be confined, at present, to the separation of the toxin from the proteins, proteoses, and peptones of the usual complex media.

The method of purifying toxins by precipitation with alum and cadmium chloride, as previously described (Eaton 1936 a, b), has been simplified by omitting the precipitation with alum and by making certain other modifications. Diphtheria toxin made on the proteose-peptone medium of Wadsworth and Wheeler (1934) and tetanus toxin, made on a meat infusion medium, may be considerably purified without appreciable alteration by a single precipitation with cadmium chloride.

For the purpose of comparing the properties of diphtheria and tetanus toxins, the toxins must be made on similar media and purified by similar methods unless the complete purity of the final products can be proved. Differences in the properties of the two toxins necessitate the use of slightly different methods

in their purification. For this reason the method applied to the purification of each toxin is described in enough detail to make clear the differences found.

PRODUCTION AND PURIFICATION OF DIPHTHERIA TOXIN

The crude toxin was produced by growing the Park-Williams no. 8 (5) strain of *Corynebacterium diphtheriae* on the proteose-peptone medium described by Wadsworth and Wheeler (1934). Details will be found in a previous paper (Eaton 1936 a). To the toxic filtrate are added sufficient sodium citrate to make the concentration 2 per cent and then $\frac{1}{5}$ volume of 5 per cent cadmium chloride solution. The pH should be about 7.0. The toxin solution should be stirred thoroughly during the addition of the cadmium chloride, and occasionally for an hour or two afterwards. The resulting precipitate is allowed to settle and the liquid decanted and filtered. The precipitate, which consists mostly of phosphates with some adsorbed impurities, is discarded. To the filtrate $\frac{1}{3}$ volume of 5 per cent cadmium chloride solution is added and the pH is adjusted to 6.0. A fine granular precipitate forms slowly. After the preparation has stood in the refrigerator overnight, the precipitate containing the toxin is collected and washed with water. The washed precipitate is eluted with 2 per cent sodium phosphate at pH 7.8 in the refrigerator for 24 hours. The resulting partially purified toxin is further purified by fractionation with ammonium sulphate and dialysis (Eaton 1936 a; Pappenheimer 1937). Separation of toxin from bacterial protein may be accomplished by the method already described (Eaton 1937).

PRODUCTION AND PURIFICATION OF TETANUS TOXIN

Tetanus toxin was produced from a strain of *Clostridium tetani* isolated in this laboratory. In previous purification experiments (Eaton 1936 b) a strain of *C. tetani* obtained from the New York State Laboratories was used for the production of toxin. The cultures were grown in two-liter Erlenmeyer flasks filled almost to the neck and sealed with vaseline. The toxin was filtered after incubating the cultures for two weeks. The medium consisted

of the ordinary veal infusion broth containing 2 per cent of Bacto-peptone and 1 per cent of glucose.

The filtrate is treated with $\frac{1}{20}$ volume of 20 per cent calcium chloride solution and filtered to remove phosphates. To the filtrate adjusted to pH 6.7 to 7.0 is added sodium chloride to a concentration of 5 per cent and then $\frac{1}{2}$ volume of 5 per cent cadmium chloride solution. If no precipitate forms, the pH of the solution is raised by the addition of N/1 NaOH until a slight turbidity appears. If the precipitate is very heavy, the pH should be lowered (see next section). After standing overnight the precipitate is collected, washed, and eluted as described for diphtheria toxin.

The eluted toxin is precipitated by ammonium sulphate in the fraction between 0.4 and 0.6 saturation. The ammonium sulphate may be removed from the redissolved precipitate by dialysis in a cellophane bag. In the attempts to further purify tetanus toxin by fractionation with ammonium sulphate and dialysis much of the toxin was lost. This is apparently due to the instability of the purified toxin.

For the determination of the M.L.D. of crude and purified tetanus toxin, broth was used for a diluent because the toxicity could not be measured satisfactorily by diluting the toxin in saline. Halter (1936) and others have observed that crude tetanus toxin diluted in saline is less stable than when it is diluted in broth. This is probably attributable to a protective effect of the broth, not to the activation of the toxin by anything in the broth. Bronfenbrenner (1924) has made similar observations with botulinus toxin.

THE EFFECT OF SODIUM CITRATE, SODIUM CHLORIDE, AND pH ON PURIFICATION OF TOXINS BY PRECIPITATION WITH CADMIUM CHLORIDE

Cadmium chloride alone added to crude toxin or the sterile culture medium precipitates not only proteins but also proteoses and possibly other polypeptides. The amount and character of the material precipitated depends on the pH. In a solution containing 2 per cent sodium citrate or 5 per cent sodium chloride,

the compounds formed between cadmium chloride and the nitrogenous substances are more soluble, and an excess of the reagent is required to produce precipitation. Under these conditions the solubility of the nitrogenous impurities is relatively greater than the solubility of the toxic fraction. The character of the material precipitated depends on the amount and kind of salt present (sodium chloride or sodium citrate), the concentration of cadmium chloride, and the pH. In preliminary experiments tetanus toxin was precipitated by cadmium chloride in the presence of 2 per cent sodium citrate. Later, it was found that better yields of purer toxin were obtained by using 5 per cent sodium chloride in place of the citrate.

With both tetanus and diphtheria toxins the higher the pH the more precipitate is formed, and the more impure is the resulting preparation. Maximum purification is obtained by precipitating the toxin at the lowest pH consistent with a reasonable yield of pure toxin. For diphtheria toxin the optimum pH is near 6.0. For tetanus toxin the optimum pH is higher, generally between 6.3 and 7.0. Since this varies with different lots of toxin, the pH of precipitation with maximum yield and purification should be determined in small samples before precipitating the bulk of the toxin.

RESULTS OF THE PURIFICATION PROCESS

Diphtheria and tetanus toxins were purified to about the same degree by the methods just described, and the yields of toxin were similar in each case (table 1). In terms of nitrogen per M.L.D., diphtheria toxin purified by precipitation with cadmium chloride is not as pure as the best preparations previously obtained by more complex procedures from crude toxins prepared on the same medium, but its purity compares favorably with that of toxin purified by any procedure involving only one precipitation. By fractionation with ammonium sulphate and dialysis, impurities in the form of inactive protein and proteoses are separated from the toxin and the resulting preparation contains only slightly more nitrogen per M.L.D. than the purest diphtheria

toxin so far obtained. The tetanus toxin purified by cadmium precipitation is at least twice as active as the preparation previously obtained by a more complex procedure (Eaton 1936 b). As indicated in the last two lines of table 1, fractionation of the purified tetanus toxin with ammonium sulphate and dialysis resulted in a marked reduction of the yield, and little further purification was accomplished. Part of the loss of toxin occurred during the precipitation with ammonium sulphate, and 50 to 75 per cent was lost during dialysis in cellophane.

TABLE 1

Results of the purification of tetanus and diphtheria toxins

TOXIN	PURIFICATION PROCEDURE	N PER M.L.D.	TEST ANIMAL	PURIFICATION FACTOR	YIELD
		mgm.			*per cent*
Diphtheria	Crude	0.00520	Guinea pig		
Diphtheria	cd	0.000100	Guinea pig	52	75
Diphtheria	cd	0.000044	Guinea pig	120	65
Diphtheria	cd., A.S.	0.000028	Guinea pig	186	60
Tetanus	Crude	0.000250	Mouse		
Tetanus, Lot A	cd	0.0000057	Mouse	44	80
Tetanus, Lot B	cd	0.0000020	Mouse	125	90
Tetanus, Lot A	cd., A.S.	0.0000041	Mouse	61	10
Tetanus, Lot B	cd., A.S.	0.0000018	Mouse	138	22

Purification procedure: cd, precipitation with cadmium chloride as described in text; A.S., fractionation with ammonium sulphate.

Nitrogen determined by the micro-Kjeldahl method of Pregl.

$$\text{Purification factor} = \frac{\text{Mgm. N/M.L.D. in crude toxin}}{\text{Mgm. N/M.L.D. in purified toxin}}.$$

$$\text{Yield} = \frac{\text{Total M.L.D. in purified toxin}}{\text{Total M.L.D. in crude toxin}}.$$

THE COMPOSITION OF TETANUS TOXIN COMPARED WITH DIPHTHERIA TOXIN

The results presented in table 2 indicate that tetanus toxin purified to the same degree as diphtheria toxin by a similar method contains relatively little protein. Although practically all the nitrogen in the purest diphtheria toxin is precipitable by

trichloracetic acid, only 8 to 18 per cent of the nitrogen in the preparations of purified tetanus toxin is precipitable as protein. If the 8 per cent of protein in one of the preparations were all toxin, the lethal dose of this protein for mice would be 0.0000000008 gram, an almost unbelievably small figure. By fractionation with ammonium sulphate and dialysis the toxicity and non-protein nitrogen are reduced and the protein nitrogen is increased to 35 per cent. This does not necessarily mean that the toxin is associated with the non-protein fraction which diffuses through the membrane. It is possible that the activity of the protein is destroyed without appreciably affecting its chemical

TABLE 2

Total nitrogen and protein nitrogen in purified preparations of diphtheria and tetanus toxins

TOXIN	PURIFICATION PROCECURE	M.L.D. PER CC.	TOTAL N PER CC.	PROTEIN N PER CC.	PROTEIN N IN TOTAL
			mgm.	*mgm.*	*per cent*
Tetanus, Lot C	cd	35,000	0.095	0.018	18
Tetanus, Lot B	cd	40,000	0.078	0.005	8
Tetanus, Lot A	cd	40,000	0.235	0.014	10
Tetanus, Lot A	cd., A.S.	8,000	0.033	0.011	35
Diphtheria	cd., A.S.	6,000	0.157	0.118	75*

Protein nitrogen is the nitrogen in the precipitate produced by 5 per cent trichloracetic acid.

* In more highly purified preparations of diphtheria toxin the protein nitrogen approaches 100 per cent.

properties. Sixty to 80 per cent of the nitrogenous material in the preparations of purified tetanus toxin is precipitable by tannic acid. This indicates that a large proportion of the nitrogen is in proteose or polypeptide form. The remainder of the nitrogen (20 to 40 per cent) is in substances of unknown nature.

In the preparations of purified tetanus toxin obtained by a different method from a different strain of *C. tetani* (Eaton 1936 b) 25 to 50 per cent of the nitrogenous material was precipitated by trichloracetic acid as protein. Those preparations were less pure than the ones described in the present paper, and it is possible that they contained more inactive protein.

THE EFFECT OF ACID ON TETANUS AND DIPHTHERIA TOXINS

Like many proteins, diphtheria toxin is precipitated by lowering the pH of the solution. The isoelectric point of the toxin is near pH 4.1 (Pappenheimer 1937). It is likely that the precipitation of diphtheria toxin by acid is in part caused by denaturation of the protein which makes it less soluble (Eaton 1937). Several samples of tetanus toxin, purified by precipitation with cadmium chloride and fractionation with ammonium sulphate, were brought to pH 4.2 to 4.4 by the addition of dilute hydrochloric acid. A slight precipitate formed and was centrifuged down and redissolved in phosphate buffer at pH 7.0. The supernatant, adjusted to neutrality, and the redissolved precipitate were then tested for toxicity. It was found in all samples tested that at least 80 per cent of the toxin remained in the supernatant. The toxin found in the precipitate was probably carried down by adsorption to inactive protein.

Attempts to precipitate the toxin at a lower pH were unsuccessful. No further precipitate was formed when the solution of purified toxin was gradually acidified down to a pH of 2.4. At this pH the toxin is quite rapidly destroyed. Addition of 5 per cent trichloracetic acid (pH about 1.2) causes precipitation of some material which remains in solution at pH of 4.2, but the toxin is, of course, destroyed by the strong acid. These results indicate that tetanus toxin, when considerably purified, is not precipitated by acid at any pH at which the toxin is stable. Sommer (1937), Boivin and Izard (1937), and Ramon, Boivin, and Richou (1937) have purified tetanus toxin and anatoxin by precipitation with acid. It is probable that in these experiments the toxin was adsorbed to inactive protein precipitated by the acid.

PRECIPITATION OF PURIFIED TOXINS BY ALUM

Purified diphtheria toxin is readily precipitated by ammonium aluminium sulphate or ferric ammonium sulphate between pH 6.0 and 7.0. Tetanus toxin is precipitated almost completely from crude filtrates by ferric ammonium sulphate and incompletely

by ammonium aluminium sulphate. After purification by the procedure described in this paper tetanus toxin is not readily precipitated by either reagent. The toxin was completely precipitated by ferric ammonium sulphate from one preparation, but in other experiments more than half of the toxin remained in the supernatant. By adding enough phosphate to cause the formation of a voluminous precipitate, more toxin could be brought down. These results suggest that tetanus toxin is precipitated only by adsorption to precipitates containing protein and insoluble phosphates.

DISCUSSION

Preparations of diphtheria and tetanus toxins purified to about the same degree by similar chemical procedures differ in the relative proportions of protein and non-protein nitrogen they contain, as well as in other chemical properties. Cadmium chloride under certain conditions precipitates proteoses as well as proteins. This accounts for the fact that one preparation is composed predominantly of protein and the other mostly of proteose or polypeptide.

The optimum conditions for the purification of the toxins were worked out empirically by trying various combinations of the reagents at different concentrations and values of pH and not by any preconceived idea as to the nature of the active fraction to be precipitated. As a result, the conditions for precipitation have been adjusted so that the most active diphtheria toxin is precipitated as a protein by cadmium chloride at a pH of 6.0 while tetanus toxin of maximum activity per mg. of nitrogen is precipitated in a slightly more alkaline pH range where proteoses are precipitated. The degree of purification attained indicates that the precipitation of both toxins is quite selective. It is likely that similar combinations of sodium citrate, sodium chloride, and cadmium chloride or other metallic salt could be used to selectively precipitate other proteins or polypeptides from broth filtrates, but the best combinations would not be the same in every case.

The adsorption of toxins, viruses, and other highly active agents to inactive precipitates is a well known phenomenon. This does

not mean that all precipitates containing these agents are adsorption complexes. It is often possible to distinguish between adsorption and true precipitation of a chemical compound. Diphtheria toxin may be precipitated under relatively constant conditions by a variety of protein precipitants such as ammonium sulphate, phosphotungstic, or nucleic acids, lead acetate, uranyl acetate, potassium aluminum sulphate, acetone, or alcohol (Eaton 1936 a). The fact that highly purified diphtheria toxin behaves in the same way toward these reagents as toxin in the crude state indicates that the toxin itself is precipitated and not merely adsorbed to precipitates of inactive protein. Highly purified tetanus toxin differs from crude toxin in its behavior toward acid, alum, and possibly several other protein reagents. This indicates that adsorption to protein precipitates plays a part in the precipitation of tetanus toxin by protein reagents. The purified toxin is no longer precipitable by reagents which partially or completely precipitate it from crude filtrates. Diphtheria and tetanus toxins are salted out at approximately the same concentration of ammonium sulphate either in the crude or purified state.

Generalizations about the nature of bacterial toxins from one or two examples are not justified. Although the characterization of diphtheria toxin as a protein has apparently been well established, tetanus toxin may not be a true protein, and the scarlatinal toxin which is resistant to tryptic digestion may prove to be a nitrogenous carbohydrate (Hooker and Follensby 1934; Stock 1937). The diversity in the nature of toxins is, of course, limited by the number of substances that will act as antigens. At the present time the only pure substances which have been clearly shown to be antigens are proteins and certain complex carbohydrates. The possibility that tetanus toxin is a carbohydrate is apparently excluded by the fact that this toxin is destroyed by tryptic digestion.

SUMMARY

A simplified method for the purification of diphtheria and tetanus toxins by precipitation with cadmium chloride is described.

The preparations of the two toxins purified to about the same degree (separation of over 99 per cent of nitrogenous impurities) differ in the proportions of protein and non-protein nitrogen present. Although diphtheria toxin is precipitated by a variety of protein reagents, purified tetanus toxin is not precipitable by acid or alum.

The findings indicate that tetanus toxin differs in its chemical properties from diphtheria toxin which is a typical protein.

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OXIDATIONS PRODUCED BY HEMOLYTIC STREPTOCOCCI

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Very little is known about the oxidations produced by hemolytic streptococci. Fujita and Kodama (1935) reported on the oxidation of glucose by *Streptococcus hemolyticus* and (because it lacked cytochrome and was cyanide insensitive) concluded that the respiration was catalyzed by Warburg and Christian's (1932) yellow ferment (phosphoriboflavin protein). Farrell's paper (1935) need not be discussed. He simply observed the reduction of a dye (indigotetrasulfonate) in the absence of oxygen, and from those observations drew conclusions on "dehydrogenations." The orientation of the reaction of an oxidizable substance may differ in the presence and in the absence of oxygen (for example, pyruvic acid, which is oxidized in the presence of oxygen, may split by dismutation in the absence of oxygen); reversible dyes may inhibit a dismutation in the absence of oxygen (Krebs (1937)); and, finally, these dyes may act as mediators of coupled oxidation-reductions, the dye being alternately reduced and oxidized. In such cases the observer would conclude by stating that no "dehydrogenation" took place. The species of bacteria grouped under the name hemolytic streptococci are of extreme interest. So far the group has defied classification by fermentation tests (Burger, 1907; Kendall, Day, Walker and Ryan, 1919; McClachlan, 1927). The rate of glucose breakdown by different strains of streptococci was also found to vary greatly when the bacteria were grown in broth containing glucose and phosphate (Hewitt, 1932).

A study of the oxidations produced by seven strains of hem-

olytic streptococci reported in this paper shows that different strains of hemolytic streptococci exhibit different metabolism; furthermore, that one strain may, on repeated transplantation in a blood-agar medium, change its oxidative enzymes.

EXPERIMENTAL

Seven strains of hemolytic streptococci were used in these experiments. They will, later on, be referred to only by the numbers given here:

No. 1. Erysipelas strain, Mosewitch; isolated in 1935, and since then subdivided into *1a*, the bacteria as studied during the first ten weeks of weekly transplant, and *1b*, the same bacteria later on.

No. 2. Scarlet fever strain, No. 2; isolated in 1924.

No. 3. Erysipelas strain, Valentine; isolated in 1934.

No. 4. Scarlet fever strain, Tyler; isolated in 1924.

No. 5. Scarlet fever strain, Greenwood; isolated in 1936.

No. 6. Scarlet fever strain, Wadsworth; isolated in 1924.

No. 7. Septicemia, Terihaj; isolated in 1937.

Most of these bacteria were isolated by Dr. George F. Dick.

After isolation, these streptococci were preserved in blood agar in sealed tubes, at room temperature. For the experiments reported here, they were grown in a beef-infusion blood-agar medium without glucose, in flat medicine bottles. After 24 hours' incubation at 37°, the bacteria were suspended in 0.9 per cent sterile NaCl, centrifuged, and suspended again in 0.9 per cent NaCl. The oxygen consumption and CO_2 production were measured at 38° by the usual Warburg-Barcroft manometric method. Two cubic centimeters of the bacterial suspension plus 0.5 cc. of 0.2 M phosphate buffer, pH 7.0, were used for each vessel. All these strains of hemolytic streptococci consumed negligible quantities of oxygen in the absence of oxidizable substances; thus, in every case, the vessel used as thermobarometer contained the bacterial suspension plus the buffer. The oxidizable substrates were kept in the side-arm of the Warburg vessels and were poured into the main vessel containing the bacterial suspension once temperature equilibrium was reached.

A pH value of 7.0 was chosen because this was found to be the optimum, on studying the influence of hydrogen ion concentration on the rate of oxidation of glucose, lactic acid, pyruvic acid, and glycerol by all the strains of streptococci used in these experiments. Volatile acids were determined by Friedemann's method (1937) adapted for the determination of small volumes. All the experiments reported here were repeatedly verified during a period of one year. Every bottle containing the bacteria was carefully examined before the experiments, those bottles which showed accidental contamination being rejected. The Warburg vessels, as well as all the pipettes used, were sterilized by keeping them overnight at 110°. The experiments never lasted more than two hours, at the end of which time smears were made from the bacterial suspensions in the Warburg vessels to verify lack of contamination.

THE OXIDATION OF CARBOHYDRATES

Extended experiments on the oxidation of carbohydrates were conducted, mainly with Streptococcus No. 1a. The hexoses were rapidly oxidized, the rate of oxidation decreasing in the following order: glucose, levulose, mannose, galactose. Arabinose was oxidized very slowly. Lactose was oxidized at a rate one-third that of glucose. Glyceric aldehyde was oxidized at about the same rate as lactose. Hexose monophosphate was oxidized slowly.[1] In table 1 are given the relative rates of oxidation of these carbohydrates, calculated by taking the rate of oxidation of glucose as 100. In all these experiments, the amount of oxidizable substrate was 0.03 m Mole, and the oxygen uptake was measured for one hour.

Oxidation of glucose by the different strains of hemolytic streptococci

In the absence of oxygen, glucose is fermented by streptococci mainly to lactic acid (Hewitt 1932; Friedemann).[2] In the presence of oxygen, washed streptococci oxidized glucose partially.

[1] The sample of hexose monophosphate used in these experiments was kindly sent to one of us by Dr. C. V. Smythe.

[2] Personal communication by Dr. T. H. Friedemann.

TABLE 1

Oxidation of carbohydrates by Streptococcus hemolyticus no. 1a

As a rule, 1 mgm. of dry bacteria consumed 126 c.mm. O_2 per hour with glucose as substrate. Temp., 38°; pH, 7.01. Relative rate calculated by taking the rate of glucose oxidation as 100.

CARBOHYDRATE	RELATIVE RATE OF OXIDATION
Glucose	100
Levulose	92
Mannose	83
Galactose	77
Glyceric aldehyde	31
Lactose	30
Hexose monophosphate	13
Arabinose	1.3

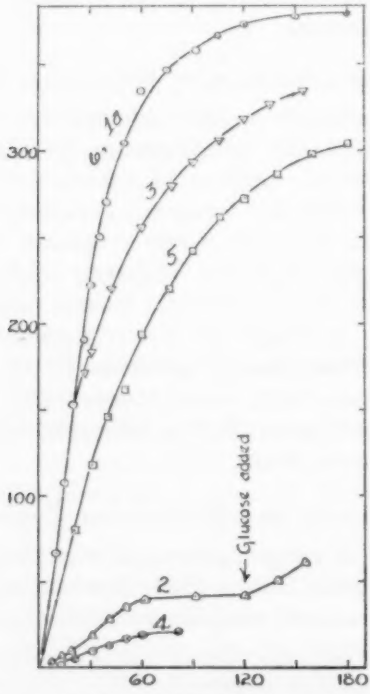

FIG. 1. OXIDATION OF GLUCOSE BY HEMOLYTIC STREPTOCOCCI

Amount of glucose, 0.01 mMole, Temp. 38°; pH, 7.01. Abscissa, time in minutes; ordinate, O_2 uptake in c.mm. The numbers indicate the streptococcus species as numbered in the text.

Streptococci Nos. 1a, 3, 5, and 6 showed a rapid oxygen consumption up to 1.5 moles of oxygen per mole of glucose, the rate of oxidation becoming then gradually slower. Streptococci Nos. 2 and 4 oxidized glucose quite slowly, so slowly that the experiments had to be stopped before there was consumption of even 0.5 mole of oxygen per mole of glucose. The end of glucose oxidation can not be due to the accumulation of inhibitory oxidation products (as with pneumococci it is due to H_2O_2 accumulation), for the addition of more glucose started the oxygen uptake at the same rate as initially (fig. 1).

A glance at figure 1 shows that the strains here studied may be divided into two groups: those which oxidize glucose rather rapidly up to 1.5 moles of oxygen per mole of glucose; and those which oxidize glucose very slowly, the oxidation stopping with about $\frac{1}{2}$ mole of oxygen per mole of glucose. It must be emphasized that this difference in rates of oxidation is not due to the influence of environmental conditions, but rather to the characteristics of the bacteria, because the optimum pH value for the oxidation of glucose was found to be between 6.8 and 7.1 (the experiments were performed at a pH value of 7.0).

Effect of NaF and CH_2ICOOH on the oxidation of glucose

Glucose utilization by living cells seems to proceed according to varied mechanisms. Thus, the series of phosphorylations which occur in muscle during glycolysis do not occur in embryonic tissues, according to Needham and coworkers (1937). Furthermore, glucose may be oxidized either after its transformation to the C_3 compounds or after its transformation to hexose phosphates; it may even be oxidized directly. NaF (0.02 M) and iodoacetic acid (0.01 M), the well known inhibitors of glucose fermentation, inhibited the oxidation of glucose, the first completely, the second to the extent of 95 per cent (table 2). These inhibitions may be taken as indication that in the case of hemolytic streptococci glucose is first transformed into a triose or a hexose phosphate before it is oxidized. (It has been shown by Embden and Deuticke (1934) that the NaF inhibits the splitting of phosphoglyceric acid and that CH_2ICOOH inhibits the con-

version of hexosediphosphate into the triose phosphates.) This inhibiting effect was observed in all the strains here reported.

Effect of HCN on the oxidation of glucose

Burnet (1927) was the first to report that *Streptococcus hemolyticus* could be grown in the presence of cyanide; he classified the bacteria as cyanide insensitive. His observations were confirmed by Fujita and Kodama (1935). The effect of HCN on the oxidation of glucose by hemolytic streptococci was found to vary according to the strain. In strains No. 1a, 5, and 6 the oxidation

TABLE 2

Effect of inhibitors on the oxidation of glucose by hemolytic streptococcus

INHIBITOR	O_2 UPTAKE IN 30 MINUTES		PER CENT INHIBITION
	Control	With inhibitor	
	c.mm.	c.mm.	
NaF (0.02 M) Strain No. 1	130.0	0	Complete inhibition
CH_2ICOOH (0.01 M) Strain No. 1	126.0	6.5	95
HCN (0.01 M) Strain No. 1a (April 23, 1937)	127.3	124.5	No inhibition
HCN, Strain No. 4	148.5	140.0	5.7
HCN, Strain No. 1b (November 11, 1937)	98.0	50.8	48.0
HCN, Strain No. 2	63.0	50.0	20.6
HCN, Strain No. 5	253.4	250.5	No inhibition
HCN, Strain No. 6	198.0	195.5	No inhibition

of glucose was not inhibited by HCN. It was partially inhibited in strains No. 1b, 4, and 2. In all these experiments the usual care was taken in order to avoid distillation of HCN over the KOH which was used to absorb CO_2 production.

OXIDATION OF LACTIC ACID

The different oxidative behavior of various hemolytic streptococci can be better demonstrated by studying the oxidation of lactic and pyruvic acids and glycerol, for here there is a difference not only in rates of oxidation but, what is more significant, in

the number of oxidations performed. Thus, some strains of hemolytic streptococci (Nos. 1a, 3, 5) oxidized lactic acid; others (2, 7, and 1b) did not oxidize lactic acid at all. Strain No. 4 oxidized it at a very slow rate (table 3). Those strains of hem-

TABLE 3

Oxidation of lactic acid, pyruvic acid, and glycerol by hemolytic streptococci
Amount of substrates, 0.02 mM; pH, 7.0; Temp., 38°

STREPTOCOCCUS NUMBER	OXYGEN UPTAKE IN 30 MINUTES		
	Lactate	Pyruvate	Glycerol
	c.mm.	c.mm.	c.mm.
1a	208	98.4	206
2	0	5.6	129
3	34	10.5	0
4	2	4.7	1,490
5	52	0	0
6	44	7.3	15
7	0		195

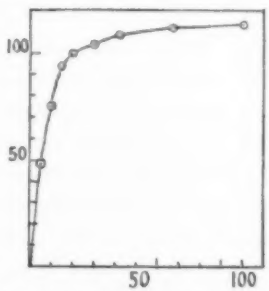

FIG. 2. OXIDATION OF LACTATE BY HEMOLYTIC STREPTOCOCCUS NO. 1a
Temp., 38°; pH, 7.01. Amount of lithium lactate, 0.01 mMole. Abscissa, time in minutes; ordinate, O_2 uptake in c.mm. All strains of streptococci which oxidized lactate behaved similarly.

olytic streptococci which did oxidize lactic acid at a measureable rate (Nos. 1a, 3, 5) used up one-half mole of oxygen per mole of lactic acid (fig. 2). These experiments could be interpreted as an oxidation of lactate to pyruvate:

$$CH_3CHOHCOOH + \tfrac{1}{2}O_2 \rightarrow CH_3COCOOH + H_2O$$

A determination of the $\frac{CO_2 \text{ output}}{O_2 \text{ uptake}}$ quotient, however, showed that the lactate oxidation was accompanied by O_2 uptake and CO_2 output. 0.01 millimol of lactate used up on its oxidation 131 c.mm. of oxygen and produced 127.1 c.mm. CO_2. A respiratory quotient of one is obtained when lactic acid is oxidized to acetic acid and CO_2:

$$CH_3CHOHCOOH + O_2 \rightarrow CH_3COOH + CO_2 + H_2O$$

After steam distillation, the volatile acid distilled was identified as acetic acid by Krüger and Tschirch's (1929) color reaction with lanthanum nitrate and iodine. No formic acid or pyruvic acid was found. It may therefore be concluded that lactic acid is oxidized directly to acetic acid and CO_2. The factors responsible for the incomplete oxidation of lactic acid were not investigated.

Effect of inhibitors and dyes on the oxidation of lactic acid

Barron and Hastings (1933), on studying the oxidation of lactic acid by gonococci, found this oxidation performed through the coöperation of two factors: an oxidizing catalyst, probably a hemochromogen, and an activating enzyme. The first could be inhibited by HCN or H_2S and then replaced with a reversible dye of suitable oxidation-reduction potential. The second could be inhibited by a number of narcotics. The oxidation of lactic acid by streptococci was little affected by any of these inhibiting agents. Furthermore, the reversible dyes which increased lactic acid oxidation by gonococci had no effect or even inhibited its oxidation by hemolytic streptococci. Neither hydroxymalonic acid nor sodium arsenite inhibited the oxidation (table 4). In the last column of this table is given the inhibition produced by these substances on the oxidation of lactate by gonococci as found by Barron and Hastings (1933). It must be concluded from these experiments that the lactic-acid oxidizing enzyme of hemolytic streptococci is different from that usually found in other bacteria (gonococci, staphylococci, *Escherichia coli*).

OXIDATION OF PYRUVIC ACID

Streptococci No. 1 (both 1a and 1b) were the only strains which oxidized pyruvic acid rapidly. The other species of streptococci either oxidized it too slowly (Nos. 2, 3, 4, and 6) or did not oxidize it at all (No. 5) (table 3).

TABLE 4

Effect of inhibitors on the oxidation of lactate by Streptococcus hemolyticus

INHIBITOR	O_2 UPTAKE IN 30 MINUTES		INHIBITION	INHIBITION ON LACTATE OXIDATION BY GONOCOCCI
	Control	With inhibitor		
	c.mm.	*c.mm.*	*per cent*	*per cent*
HCN, 0.01 M	199.0	187.6	6.7	Complete
Phenyl urea, sat	190.0	117.0	38.4	52.0
Valeronitrile, 0.05 M	190.0	100.0	27.9	73.3
Hydroxymalonate, 0.05 M	190.0	137.0	8.7	
CH_2ICOOH, 0.01 M	68.8	62.9	8.7	62.0
Pyocyanine, 0.00015 M	133.1	118.0	11.3	Increase
Cresyl blue, 0.00015 M	150.0	79.0	47.3	Increase
Indigo tetrasulfonate, 0.00015 M	150.0	100.5	33.0	Increase
Sodium arsenite, 0.01 M	175.2	180.0	0	Increase
Octyl alcohol, sat	223.2	146.4	34.4	Complete

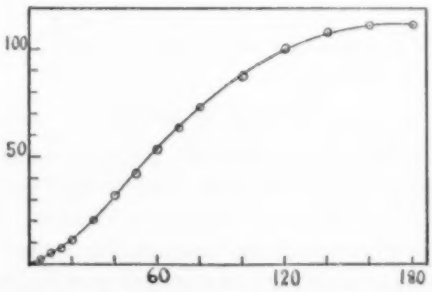

FIG. 3. OXIDATION OF PYRUVATE BY HEMOLYTIC STREPTOCOCCI, STRAIN 1a
Temp., 38°; pH, 7.01. Amount of lithium pyruvate, 0.01 mMole. Abscissa, time in minutes; ordinate, O_2 uptake in c.mm.

The oxidation of pyruvic acid by hemolytic streptococci, like that produced by gonococci (Barron (1936)), ended with an uptake of ½ mole of oxygen per mole of pyruvate (fig. 3). Since

there was an output of 1 mole of CO_2 per mole of pyruvate, the oxidation can be expressed as follows:

$$CH_3COCOOH + \tfrac{1}{2}O_2 \rightarrow CH_3COOH + CO_2$$

The validity of this equation was further demonstrated by titration of the acetic acid produced and its identification. One mole of acetic acid was formed per mole of pyruvic acid used by the bacteria. The metabolism of pyruvic acid by hemolytic streptococci follows a different path in the absence of oxygen. In such a case, as will be shown elsewhere, pyruvic acid splits into acetic acid and formic acid

$$CH_3COCOOH + H_2O \rightarrow CH_3COOH + HCOOH$$

Acetoacetic acid was not oxidized by hemolytic streptococci.

TABLE 5

Effect of inhibitors on the oxidation of pyruvate by Streptococcus hemolyticus

INHIBITOR	O_2 UPTAKE IN 30 MINUTES		INHIBITION	INHIBITION ON LACTATE OXIDATION BY GONOCOCCI
	Control	With inhibitor		
	c.mm.	*c.mm.*	*per cent*	*per cent*
HCN, 0.01 M	135.8	71.7	70	95
NaF, 0.01 M	135.8	57.2	70	75
2,6-Dichlorophenol indophenol, 0.0001 M	128.5	80.7	40.6	92

Effect of inhibitors

The oxidation of pyruvic acid by gonococci was inhibited by HCN, NaF, some reversible dyes, and a number of substances known as inhibitors of chain reactions (Barron (1936)). The oxidation of pyruvate by Streptococcus No. 1 was also inhibited by these substances, although to a less degree (table 5).

OXIDATION OF GLYCEROL

As in the case of the other two metabolites, some strains of hemolytic streptococci oxidized glycerol quite vigorously (Nos. 1b, 2, 4, and 7); Strain No. 6 oxidized it very slowly; Strains Nos. 3 and 5 failed to oxidize glycerol at all (table 3). Those

strains which did oxidize glycerol, oxidized also ethyl, propyl, and butyl alcohol; methyl alcohol, glycol, mannitol, erythritol, and sorbitol were not oxidized.

The glycerol-oxidizing enzyme in these bacteria was found to be the most stable of all oxidizing enzymes. Suspensions of washed bacteria in 0.9 per cent NaCl plus a few drops of toluol could be kept at 3° for as long as 7 days without destruction of

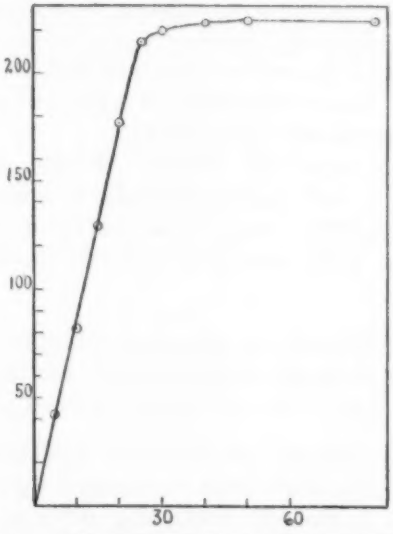

FIG. 4. OXIDATION OF GLYCEROL BY HEMOLYTIC STREPTOCOCCI, STRAIN 1a

Temp., 38°; pH, 7.01. Amount of glycerol, 0.01 mMole. Washed bacteria, kept at 3° for 30 hours. Abscissa, time in minutes; ordinate, O_2 uptake in c.mm. The total CO_2 output was 110 c.mm. $RQ = 0.5$. All the strains which oxidized glycerol behaved similarly.

the glycerol-oxidizing enzyme. These suspensions of toluol-treated bacteria oxidized glycerol rapidly with an oxygen uptake of 1 mole per mole of glycerol (fig. 4) and a CO_2 output of 0.5 mole. No acid was formed by the oxidation of glycerol; H_2O_2 formation was detected by its color reaction with titanous sulfate. There was no acetaldehyde formation. On titration with Campbell's method (1926) for the determination of dihydroxyacetone (which is not specific because glyceraldehyde can also be titrated

with phosphomolybdic acid reagent) there was found 0.5 mole per mole of glycerol oxidized. It is therefore likely that glycerol is oxidized by streptococci according to the following equation:

$$2\,CH_2OHCHOHCH_2OH + 2\,O_2 \rightarrow CH_2OHCOCH_2OH + CH_2OHCH_2OH + CO_2 + H_2O_2 + H_2O$$

An alcohol was found in the oxidation product, but whether it is glycol or not could not be established, since there are no methods to titrate glycol in the presence of other alcohols.

The oxidation of glycerol was not inhibited by HCN (0.01 M); it was inhibited almost completely by NaF (0.01 M), CH_2ICOOH (0.01 M) and pyrophosphate (0.025 M).

Formic, acetic propionic, butyric, succinic, oxalic, glycolic, citric, glyceric, α- and β-glycerophosphoric acids were not oxidized at a measurable rate by hemolytic streptococci. The following amino acids were not oxidized: alanine, asparagine, Na glutaminate.

VARIATIONS IN THE RATE OF OXIDATION OF GLUCOSE, GLYCEROL, AND LACTATE BY STREPTOCOCCUS HEMOLYTICUS NO. 1 AT DIFFERENT PERIODS

Burger (1907) pointed out that the fermentative power of a strain of streptococcus could be changed by repeated transplantation into a medium containing different sugars. This ability of bacteria to modify the nature of their enzymes when grown in the presence of special substrates has indeed been utilized by Dubos and Avery (1931) to develop bacteria able to split the capsular polysaccharides of Type III pneumococcus, and by Miller and Dubos (1936) to adapt certain bacteria to the decomposition of creatinine. Streptococcus No. 1 changed its oxidative properties while being grown in the same nutrient media, as shown in a study of the rates of oxidation of glucose, glycerol, and lactic acid at different periods from June 17, 1937, to February 6, 1938. The cultures were grown weekly in the usual medium, blood agar, and the experiments were performed as previously with 24 hour cultures. At first the rates of oxidation decreased in the following order: lactate > glucose > glyc-

erol. Gradually the bacteria lost the power of oxidizing lactic acid while their power to oxidize glycerol increased. On August 26, i.e., after ten transplantations in the blood-agar medium, these streptococci lost entirely the power to oxidize lactic acid, while retaining the power to oxidize glucose and glycerol. From that date on, the oxidizing enzymes of these bacteria remained unchanged. In figure 5 the relative oxidizing power of Streptococcus No. 1 towards glucose, glycerol, and lactate has been plotted, the rate of glucose oxidation having been taken as 100.

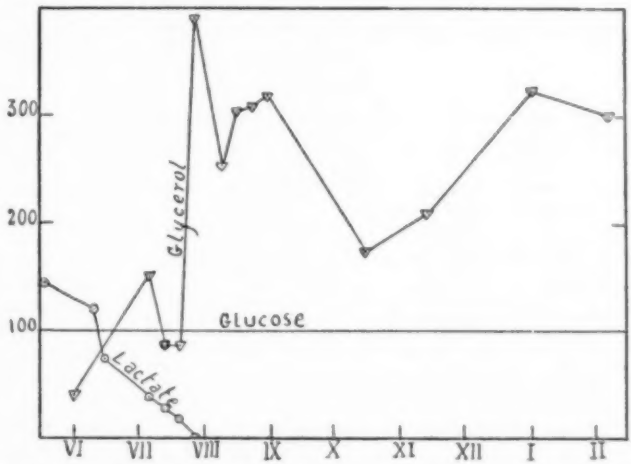

FIG. 5. RELATIVE RATES OF OXIDATION OF GLUCOSE, GLYCEROL, AND LACTATE BY STREPTOCOCCUS HEMOLYTICUS NO. 1, AS DETERMINED DURING A PERIOD OF SEVEN MONTHS

The rate of glucose oxidation has been taken as 100. Abscissa, time in months. Ordinate, relative rates of oxidation.

This change in oxidation enzymes was not confined to the loss of the lactic acid oxidizing enzyme, but extended to the mechanism of glucose oxidation. Thus the bacteria during the first weeks (Streptococcus No. 1a) appeared to oxidize glucose via lactic acid with acetic acid and CO_2 as end products, according to the equation:

$$C_6H_{12}O_6 \xrightarrow{\text{fermentation}} 2\,C_3H_6O_3;$$

$$2\,C_3H_6O_3 + 2\,O_2 = 2\,C_2H_4O_2 + 2\,CO_2 + 2\,H_2O$$

The oxidation of glucose used up 2 moles of oxygen per mole of glucose, with a CO_2 production of 2 moles; furthermore, the rate of lactate oxidation was greater than that of glucose. When the streptococci lost the power of oxidizing lactate, the oxidation of glucose ended with an oxygen uptake of $\frac{1}{2}$ mole per mole of glucose. The renewal of oxygen uptake upon the addition of more glucose shows that this process was stopped by lack of oxidizable substance and not by death of the bacteria (fig. 6).

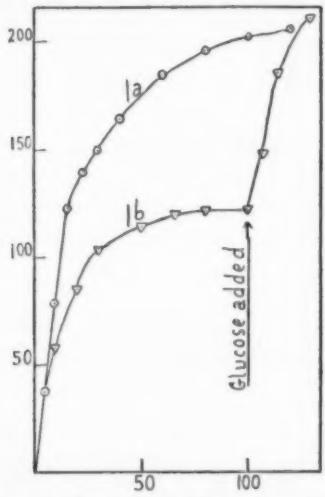

FIG. 6. THE OXIDATION OF GLUCOSE BY STREPTOCOCCUS HEMOLYTICUS NO. 1
Ia. During the first ten weeks of weekly transplantation in blood-agar. *Ib*. After seven months of frequent transplantations. Abscissa, time in minutes; ordinate, O_2 uptake in c.mm.

The first oxidation was insensitive to HCN; the second was partially inhibited by HCN (0.01 M).

It must be emphasized that the loss of the ability to oxidize lactic acid, the fundamental change, did not occur *suddenly* but *gradually*, and that the streptococci retained their pathogenic power as shown in tests with mice. An objection to the theory of strain change might be the assumption that the original culture contained two strains of streptococci, one similar to strains 1a, 3, 4, 5, and 6, able to oxidize lactic acid; and the other, similar to

strains 2 and 7, unable to oxidize it, and that in the process of repeated transplantations the latter strain became more and more dominant. This hypothesis would not explain the fact that the rate of pyruvic acid oxidation *remained unchanged* throughout the whole time the experiments were performed, namely, one year.

DISCUSSION

Oxidations produced by hemolytic streptococci, although small in number, vary greatly from one strain to another. These differences are particularly striking in comparing the oxidations

TABLE 6

Relative rates of oxidation produced by seven strains of hemolytic Streptococcus
For the calculation of these rates, the rate of glucose oxidation was taken as 100

STREPTOCOCCUS NUMBER	RELATIVE RATES OF OXIDATION			
	Glucose	Glycerol	Lactate	Pyruvate
1a (erysipelas)		40	144	35
1b (erysipelas)		174	0	36
2 (scarlet fever)	Rate too slow	642	0	26
3 (erysipelas)		0	29	3
4 (scarlet fever)	Rate too slow	1,490	25	0
5 (scarlet fever)		0	45	0
6 (scarlet fever)		7	20	1
7 (septicemia)		327	0	

produced by hemolytic streptococci isolated from cases of scarlet fever. Streptococcus No. 2 oxidized glycerol almost exclusively, for the rate of glucose and pyruvic acid oxidation was small, and lactic acid was not oxidized. Streptococcus No. 6 oxidized glucose at high speed, and lactate at lower speed; glycerol and pyruvate were oxidized very slowly. Streptococcus No. 5 oxidized only glucose and lactate; glycerol and pyruvate were not oxidized (table 6). The relative rates were calculated by making an average of all the experiments performed with these bacteria during one year. If such varied forms of oxidation are found in hemolytic streptococci isolated from scarlet fever,

it is easy to understand the failure of the numerous attempts to classify the whole group of bacteria collected under the name of hemolytic streptococci. This failure is more apparent in Streptococcus No. 1, an organism isolated by Dr. Dick in 1935 from a case of erysipelas and kept for two years in his laboratory. It changed its metabolism on repeated transplantation. An organism which oxidized lactate changed into one which did not oxidize it.

It is quite possible that these differences of metabolism may also be found in other bacteria. For example, Sevag and Neuenschwander-Lemmer (1936) reported that *Staphylococcus aureus* (grown in bouillon) did *not* oxidize pyruvic acid. Krebs (1937) found that *Staphylococcus aureus* grown in broth agar oxidized pyruvic acid to about half of complete oxidation. Barron and Lyman (to be published) on studying five different strains of *Staphylococcus aureus* found that one of them oxidized pyruvic acid to completion (CO_2 and H_2O) while the other four oxidized it readily to $\frac{2}{3}$ of completion.

It must be emphasized that, though the oxidation of lactic acid by α-hydroxyoxidase from gonococci and hemolytic streptococci ends with an oxygen uptake of $\frac{1}{2}$ mole per mole of lactic acid, the two oxidation processes are different. The first oxidation, a reversible process, ends with the formation of pyruvic acid; the second process is a direct oxidation to acetic acid and CO_2, a decarboxylative oxidation.

SUMMARY

The metabolism of hemolytic streptococci, as studied in seven different strains for a period of one year, showed a great variation in the number of oxidations produced by the bacteria, as well as in the relative rates of oxidation. One group oxidized glucose, lactic acid, pyruvic acid, and glycerol; a second group oxidized glucose, lactic acid, and glycerol; a third group oxidized only glucose and lactic acid. Furthermore one strain, on continued transplantation, lost *gradually* the power to oxidize lactic acid while retaining its pathogenicity and the power to oxidize pyruvic acid.

We express our thanks to Dr. George F. Dick for kindly giving us his collection of hemolytic streptococci for use in these experiments.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

MARYLAND BRANCH

PANEL DISCUSSION ON COLIFORM BACTERIA, MAY 10, 1938

Contributors: Harry E. Jordan, Carl J. Lauter, and C. A. Perry

ESCHERICHIA COLI VERSUS THE COLIFORM GROUP OF BACTERIA AS AN INDEX OF FECAL POLLUTION IN SHELLFISH AND THE VALUE OF THE EIJKMAN TEST AS A PRIMARY TEST FOR ESCHERICHIA COLI. *C. A. Perry*, Maryland State Department of Health.

It was pointed out that while the coliform group of bacteria offers a satisfactory test to determine the freedom of drinking water from ordinary pathogenic bacteria of the enteric group, the use of one of the simpler tests (such as brilliant-green lactose bile medium) would obviate the need for confirmation, would be sufficiently accurate and sensitive for general purposes, and would make results available earlier, while the time saved could be profitably used, when necessary, for the determination of *Escherichia coli*. Information on *Escherichia coli* pollution would be of considerable value in many small drinking water supplies which it is not practical to treat or where treatment is not warranted on the basis of sanitary inspection.

However valid the coliform group may be for drinking waters which may be filtered, chlorinated or protected, the use of the group has not been found satisfactory for estimating pollution in shellfish in which certain coliform bacteria especially of the *Aerobacter cloacae* type have a natural habitat and grow to large numbers under suitable temperatures and in the absence of pollution.

Escherichia coli has been found to correlate closely with pollution as estimated from sanitary survey data and to be a satisfactory index for estimating probable fecal pollution in shellfish.

Primary culture in a suitable medium at 45.5°C. has been found a practical test for *Escherichia coli*. Most *Citrobacter* and *Aerobacter* strains are inhibited under these conditions with the result that a great deal more *Escherichia coli* may be isolated than is possible with lactose broth at 37°C. This is particularly true where relatively large numbers of *Citrobacter* or *Aerobacter* types are present. The original Eijkman medium of 1904 has been greatly modified and a simple, effective medium developed. Lactose has recently been found superior to glucose in the Modified Eijkman medium in the recovery of *Escherichia coli* from raw sewage. It is believed that by further study this test may be made even more practical.

SOUTHERN CALIFORNIA BRANCH

(IN COOPERATION WITH PACIFIC DIVISION OF THE A. A. A. S.)
SAN DIEGO, CALIFORNIA, JUNE 24, 1938

THE RÔLE OF MARINE MICROÖRGANISMS IN THE FORMATION OF PRIMARY FILM ON SUBMERGED SURFACES. *W. Forest Whedon*, Scripps Institution of Oceanography, La Jolla, California. The work to be reported here has

been carried on by the Bureau of Construction and Repair, a division of the United States Navy Department, with the coöperation of the Scripps Institution of Oceanography, La Jolla, California.

The marine microörganisms which contribute to the formation of primary film on submerged surfaces include a great many species of bacteria, and several species of protozoa, namely, suctoria, ciliates, and amebae. Since only a comparatively few free-living bacteria occur in the sea and practically all others are associated with organic detritus, an understanding of the extensiveness of this relationship in primary film formation assumes an aspect of prime importance. The evidence presented by this investigation has shown that the products which result from the breakdown of organic detritus tend to adhere to the surface of a submerged object and to spread out over its surface. The bacteria multiply quite rapidly, following attachment, and spread out over the surface with the flow of the dissolving organic matter, thus forming a film.

THE INFLUENCE OF TEMPERATURE ON THE ACTIVITIES OF MARINE BACTERIA. *Claude E. ZoBell and Catharine B. Feltham*, Scripps Institution of Oceanography, University of California, La Jolla, California.

Marine bacteria have been found to be hypersensitive to temperature changes. Even the short exposure to the pouring temperature of nutrient agar (40 to 45°C.) during plating procedures is lethal to many marine bacteria and some lose their viability when exposed to temperatures of no higher than 30°C. for 10 minutes. The cause of the extreme heat-sensitivity is not well understood, particularly in view of the fact that within a few generations most of the marine bacteria can be acclimatized to tolerate temperatures as high as 55°C. for ten minutes.

The optimum temperature for the multiplication of these bacteria is between 15° and 25°C. That for the activity of their enzymes is between 25° and 35°C. No psychrophiles have been observed, although most of the bacteria are habitats of a marine environment which is perpetually colder than 4°C. However, nearly all of them will multiply at near zero temperatures. Some form colonies on plates incubated at −4 to −7°C. The ability of marine bacteria to grow at low temperatures accounts for the fact that marine food products may slowly undergo decomposition even at refrigeration temperatures.

PRECURSORS TO THE FORMATION OF CREATININE BY BACTERIA. *T. D. Beckwith and C. H. Fish*, Department of Bacteriology, University of California at Los Angeles.

Creatinine can be produced from peptone by a variety of bacteria but the peptone must be considered as a complex structure involving many factors. Further consideration has proven that creatinine may be formed through bacterial action from various amino acids when glucose is present. In seeking for its precursors, creatinine has been treated as a compound containing two critical portions which are (1) acetic acid and (2) guanidine. It has been shown that glycine, urea and glucose when under the influence of *Bacillus proteus* give rise to considerable concentrations of creatinine. Other amino acids than glycine supply either the acetic acid or urea necessary for the reaction. Added evidence regarding the importance of urea is presented by treatment of arginine by an activator of arginase. For production

of creatinine by *B. proteus*, urea may not be substituted by a salt of ammonium. The hexose glucose is the form of carbohydrate most readily available for use by the organism in order that creatinine may appear. The precursors of creatinine, as produced by *B. proteus*, are acetic acid and urea.

THE EFFECT OF INDOL 3 ACETIC ACID UPON MULTIPLICATION OF B. COLI-COMMUNIS AND B. TYPHOSUS. *T. D. Beckwith and E. H. Geary*, Department of Bacteriology, University of California at Los Angeles.

For these experiments the two organisms named were chosen since *Bacillus coli* produces both indole and acetic acid while *Bacillus typhosus* does not form indole from tryptophane. With the organism of typhoid, concentrations of the heteroauxin lying between 1:3,000,000 and 1:25,000 are stimulating to multiplication while with *Bacillus coli-communis* the zone of stimulation spreads from 1:3,000,000 to 1:5,000. Concentrations of 1:100 and greater were found to be completely toxic for both organisms and some growth inhibition was noted at the concentration of 1:1,000.

THE PROTECTIVE ACTION OF TYPHOID BACTERIOPHAGE ON EXPERIMENTAL TYPHOID INFECTION IN MICE. *Roy T. Fisk*, University of Southern California School of Medicine and Los Angeles General Hospital.

A GENERALIZED ASPERGILLUS INFECTION OF PENGUINS. *Leo F. Conti*, San Diego Zoological Research Laboratory, San Diego.

Advanced pulmonary Aspergillosis in Humboldt penguins (*Spheniscus humboldtii*) from Peru, found on autopsy twenty-nine days after removal from their natural habitat, may indicate the infection as originating in nature.

Deflation of all air sacs prior to death produces what erroneously appears to be a loss of weight.

The clotting time of blood in penguins has not been recorded, its rapid fibrination making the blood-counting procedure extremely difficult.

The intradermal tuberculin test reported by other workers as producing a positive reaction in pulmonary Aspergillosis of humans, was negative in penguins and an experimentally infected domestic chicken and rabbit.

Changes noted in the differential blood count of a domestic chicken artificially infected with Aspergillosis revealed an increase in the eosinophil and lymphocyte count. Leucocytosis as found in tuberculosis of chickens was observed only between the 15th and 60th days following inoculation.

Further blood studies in test animals experimentally infected with *Aspergillus fumigatus* seem warranted.

EXPERIMENTS ON ANTIRABIC IMMUNIZATION. *Anson Hoyt*, University of Southern California School of Medicine.

STUDIES ON THE NEUTRALIZING ANTIBODY IN THE SERUM OF POLIOMYELITIS PATIENTS AT ONSET AND RECOVERY. *John F. Kessel and Fred D. Stimpert*, University of Southern California School of Medicine and Los Angeles General Hospital.

COMPARATIVE METABOLISM OF THE R AND S VARIANTS OF LACTOBACILLUS PLANTARUM. *Ralph L. Tracy*, University of California School of Medicine.

FIELD CONTROL ROUTINE FOR OBTAINING SANITIZATION OF EATING AND

DRINKING UTENSILS. *R. V. Stone*, Los Angeles County Health Department.

In 1935, a sub-committee of the A. P. H. A. was appointed to study the problem of sterilization of eating utensils.

In March, 1937 the A. P. H. A. year book carried tentative standard procedures developed by the committee. Essentially these included a maximum allowable count of 50,000 colonies per ml. of dish water; a bacterial count not to exceed 500 colonies per rim of a drinking glass; a temperature of wash water from 110 to 120°F.; a rinse water of not less than 170°F. or a bath with a chlorine content of from 50 to 200 p.p.m. kept at a warm temperature.

The word "sterilize" implies an actual sterility attainment. This is seldom realized. The use of the word "sanitization" more nearly describes the objective in view.

Wash and rinse water bacterial counts occur, not infrequently, in the millions, both on the Atlantic and Pacific Coasts.

Temperatures of wash waters and rinse waters are usually too low (under 110°F.). Wash waters are surprisingly dirty—chlorine rinses too seldom used.

Data gathered by the Los Angeles County Health Department have led to the development of a practical control routine. This is not the final answer to the problem; however, it provides the best field procedure so far of real value to us. It includes three things:

a. A thermometer for checking temperatures of wash waters (a minimum of 120°F. should be demanded).

b. A sediment tester for the physical demonstration that dirty dish water *is* dirty water. (Clean water should be demanded.)

c. A chlorine tester for checking the strength of chlorine rinses (100 p.p.m. should be demanded as the minimum allowed). A chlorine rinse provides a "safety zone" which is needed when one observes the usual failure of hot water heaters to maintain efficiency during rush periods.